Effects of Dietary Fats on Mechanisms of Adipose Expansion and Function

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Abstract

Effects of Dietary Fats on Mechanisms of Adipose Expansion and Function

Allison Marie Wing

2021

Obesity, defined as the excessive accumulation of white adipose tissue (WAT), is a global pandemic and increases risk of many other pathologies. However, in vivo molecular mechanisms of adipocyte formation, or adipogenesis, during obesity and high-fat diet (HFD) consumption are still being understood. WAT can expand either through the proliferation of adipocyte precursors (APs) and their differentiation to form new adipocytes (adipogenesis) or through the increased size of adipocytes (hypertrophy). To study how dietary fats impact adipose expansion, mice were fed custom HFDs, each containing a different fat source, and adipogenesis and hypertrophy were quantified. This screen illustrated how dietary fats may bias the WAT toward expanding primarily through increased adipocyte size or number. This dietary fat screen was also instrumental in identifying oleic acid as a molecular signal inducing adipocyte precursor (AP) activation and proliferation. Oleic acid increased adipogenesis in vitro through Akt2 signaling and induction of lipogenesis. Using RNA sequencing of APs from mice fed HFD, downregulation of LXRα signaling was consistently identified in adipose tissue depots that demonstrate an adipogenic response to HFD. LXRα phosphorylation was identified as a modulator in the proliferative response to HFD, with dephosphorylation of LXRα resulting in enhanced diet-induced AP proliferation. This dietary fat screen also provided an effective tool to
investigate how expression of the adipokines, adiponectin and leptin, related to mechanisms of adipose expansion. Interestingly, VWAT expression of leptin correlated with several measures of adipocyte formation, contrasting with its typical association with hypertrophy. Finally, while studies using a lard-based HFD found increased expression of inflammatory and fibrotic markers with obesity, this screen also identified several diets that induce obesity but do not result in increased expression of these markers. This work identifies a novel mechanism to regulate diet-induced AP proliferation and highlights the need to study obesity within physiologically relevant contexts.
Effects of Dietary Fats on Mechanisms of Adipose Expansion and Function

A Dissertation
Presented to the Faculty of the Graduate School
of
Yale University
in Candidacy for the Degree of
Doctor of Philosophy

by

Allison Marie Wing

Dissertation Director: Dr Matthew Rodeheffer, PhD
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<tbody>
<tr>
<td>Adipoq</td>
<td>Adiponectin</td>
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<tr>
<td>AP</td>
<td>Adipocyte precursors</td>
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<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>AdiCre-ER</td>
<td>Cre recombinase fused to the estrogen receptor under control of Adiponectin promoter</td>
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<tr>
<td>HFD</td>
<td>High fat diet</td>
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<tr>
<td>LXRα</td>
<td>Liver X receptor α</td>
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<tr>
<td>mTmG</td>
<td>Membrane Tomato/membrane GFP reporter</td>
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<tr>
<td>MDI</td>
<td>An adipogenic cocktail consisting of insulin, dexamethasone, and methylisobutylxanthine</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>OH</td>
<td>High oleic acid</td>
</tr>
<tr>
<td>PdgfRα</td>
<td>Plate-derived growth factor receptor α</td>
</tr>
<tr>
<td>SD</td>
<td>Standard diet</td>
</tr>
<tr>
<td>SWAT</td>
<td>Subcutaneous white adipose tissue (inguinal)</td>
</tr>
<tr>
<td>T0</td>
<td>T0901317, an LXRα modulator</td>
</tr>
<tr>
<td>VWAT</td>
<td>Visceral white adipose tissue (gonadal)</td>
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<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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Chapter 1: Introduction

Obesity, defined as the expansion of white adipose tissue (WAT), is becoming more prevalent, with 39% of the global population classified as overweight or obese in 2015. The association of obesity with a variety of comorbidities, including diabetes, cardiovascular disease, stroke, and cancer, makes this trend particularly troubling. There are many hypotheses for what has driven this increase in obesity, including decreased physical activity, increased availability of food, and increased popularity of processed food. Given this concerning trend, understanding mechanisms of adipose expansion and adipose homeostasis are vital to understanding and preventing human pathologies.

In contrast to brown adipose tissue which functions in thermogenic regulation, WAT functions as an energy reservoir, storing energy-dense lipids until times of hunger or need. In times of caloric excess or deficit, the WAT may grow or shrink to provide or store triacylglycerides as needed. Regulation of these processes may be controlled by secreted hormones such as insulin, which inhibits lipolysis and promotes lipogenesis, or through neuronal signaling which also controls lipidolysis. Adipose and its size are therefore at the center of energy balance, regulating calories stored and calories burned.

In addition to its functions in energy storage and dispersal, WAT is also an endocrine organ, capable of secreting many factors and even producing some hormones that are specific to the adipose tissue, known as adipokines. Adiponectin and leptin are the classic adipokines. Adiponectin exerts a variety of functions, such as promoting insulin sensitivity and reducing inflammation. In
contrast, leptin is highly involved in energy balance, signaling satiety when secreted at high levels, decreasing in response to fasting, and promoting energy expenditure in lean subjects\textsuperscript{12–14}. A leptin mutation is even the basis for one of the most commonly used mouse model of obesity; the \textit{ob/ob} mouse exhibits dramatically increased food intake, resulting in a highly obese mouse that will lose weight dramatically upon the administration of leptin\textsuperscript{15,16}.

Adipose tissue is organized into distinct depots, with the inguinal subcutaneous (SWAT) and perigonadal visceral (VWAT) adipose being the most commonly studied in mice. These depots have many functional differences such as rates of lipolysis\textsuperscript{17}, gene expression\textsuperscript{18}, and adipogenic response to a high-fat diet (HFD)\textsuperscript{19}. Preferential expansion of these two depots has been linked to differing disease outcomes. VWAT expansion is associated with increased likelihood of cardiovascular disease\textsuperscript{20}, inflammation\textsuperscript{21}, reduced angiogenic potential\textsuperscript{22}, and diabetes risk\textsuperscript{23}. In contrast, further work is needed to establish the role of SWAT in metabolic disease. While SWAT has been shown to be weakly correlated to diabetic and cardiovascular risk compared to VWAT\textsuperscript{24}, other studies have linked this depot to a decreased risk of insulin resistance\textsuperscript{25,26}. Alternatively, rather than quantify depot size, waist-to-hip ratio is a common method that is easy to measure and takes into account both SWAT and VWAT depots. A higher waist-to-hip ratio has been used as a risk factor for cardiovascular disease and mortality\textsuperscript{27}. Given the different outcomes of expanding adipose in these depots, further work is needed to elucidate how expansion of these depots is regulated.
Mechanisms of adipose expansion

In addition to location, adipose expansion must also be considered by its cellular mechanisms. Adipose tissue can grow via an increase in adipocyte number (hyperplasia) or an increase in adipocyte size (hypertrophy)\textsuperscript{28,29}. Adipocyte hypertrophy is driven by the expansion of a single lipid droplet which can measure up to 100 µm in diameter and comprises most of the adipocyte volume\textsuperscript{30}. A major mechanism of lipid droplet expansion is the uptake of lipids, primarily by the fatty acid (FA) scavenger, CD36\textsuperscript{31}, or the insulin-sensitive FA transporter, FATP1\textsuperscript{32}. A second mechanism of lipid accumulation is the conversion of glucose to lipid via lipogenesis. This process of expansion must be balanced by lipolysis and the utilization of lipids for energy throughout the body. If the balance between lipid accumulation and usage is disrupted, the adipocyte may swell dramatically, potentially resulting in a thousand fold increase in volume and contributing to expansion of the WAT\textsuperscript{33}.

During prolonged caloric excess, hypertrophy may affect the homeostasis and function of the adipose tissue itself. Monteiro et al. used computational modeling to demonstrate that a large adipocyte may experience more physical stress and will be more likely to rupture with deleterious consequences\textsuperscript{34}. Jo et al. also use a combination of modeling and observational studies to determine that larger adipocytes were more susceptible to death after prolonged HFD consumption\textsuperscript{35}. Adipocyte death results in crown-like structures, a distinctive formation of macrophages around a dead adipocyte to clear lipid and debris. These macrophages tend to express a pro-inflammatory cytokine profile\textsuperscript{36}, linking
adipocyte size and death to tissue inflammation. This adipose inflammation has linked adipocyte hypertrophy to a variety of systemic metabolic consequences, such as insulin resistance\textsuperscript{33,37,38}, increased VLDL cholesterol\textsuperscript{39}, and hepatic steatosis\textsuperscript{40}.

In addition to inflammation, adipose hypertrophy is associated with hypoxia, as oxygen can diffuse 100-200 μm into tissue\textsuperscript{41}, less than the diameter of the largest adipocytes\textsuperscript{33}. This hypoxia can then progress to adipose tissue fibrosis, mediated by chronic activation of Hypoxia-inducible factor 1α and the secretion of collagens by APs and fibroblasts\textsuperscript{42,43}. Supporting this observation of progression to fibrosis, gene expression analysis of obese SWAT in humans has identified significant upregulation of fibrotic gene networks compared to lean, post-bariatric surgery WAT\textsuperscript{44}. However, whether this fibrotic response is detrimental or protective is still being understood. While several studies have indicated that fibrosis may limit adipocyte size, releasing this check upon hypertrophy often results in decreased inflammation and improved metabolic health\textsuperscript{45,46}. These data counter the hypothesis that hypertrophy inevitably leads to adipocyte rupture and macrophage infiltration, indicating that regulation of adipose homeostasis is a complex process and further understanding is needed.

Finally, in addition to inflammation and fibrosis, adipocyte hypertrophy is linked to changes in adipokine production. Adipocyte size and obesity have been observed to correlate negatively to plasma adiponectin concentrations\textsuperscript{47,48}. Leptin is positively correlated to adipocyte size which has been linked to its role in signaling fullness to the organism\textsuperscript{49,50}. Many of these studies examine human
WAT small sample sizes or quantify adipokine production in vitro. While important, these findings should be supplemented with other studies using larger sample sizes and quantifying adiponectin and leptin in vivo.

In contrast to adipocyte hypertrophy, which may quickly fluctuate in response to fasting and refeeding conditions\textsuperscript{51,52}, adipose hyperplasia is an enduring change, due to the multi-year lifespan of an adipocyte\textsuperscript{53}. Because adipocytes are post-mitotic, an increase in adipocyte hyperplasia occurs by the proliferation and differentiation of adipocyte precursors (APs). APs have been historically studied using the immortalized pre-adipose cell line called 3T3-L1 in vitro, which accumulates lipid when treated with an adipogenic cocktail, commonly including insulin, dexamethasone, and methylisobutylxanthine\textsuperscript{54,55,56}. 3T3-L1s cells have proven instrumental to understanding molecular pathways of adipogenesis. For example, 3T3-L1 cells were employed to identify Pparγ2 which is now known as the “master regulator” of adipogenesis\textsuperscript{57}. 3T3-L1 cells were also instrumental in identifying many other crucial transcription factors regulating adipogenesis, such as Klf4\textsuperscript{58}, Krox20\textsuperscript{59}, and Cebpα, Cebpβ, and Cebpδ\textsuperscript{60}.

However, despite the ease of culturing and differentiating 3T3-L1 cells, studying primary APs and in vivo models are still crucial to understanding of adipogenesis. The adipose tissue microenvironment contains a rich variety of cell types including mature adipocytes, neurons, endothelial cells, and immune cells all of which impact adipocyte function and formation. For example, macrophages have been shown to enhance adipogenesis in a 3D cell culture environment\textsuperscript{61}. Additionally, blocking adipocyte formation in AP implants not only inhibits
angiogenesis, but using antibodies to block angiogenesis also inhibits adipogenesis, indicating dual regulation of these processes. Finally, in addition to inter-cell crosstalk in vivo, because 3T3-L1 cells are derived from a clone, they will fail to capture the array of AP subtypes present in adipose tissue.
Therefore, in vivo studies remain important to understanding adipose tissue regulation and growth.

In 2008, Rodeheffer et al. identified a series of cell markers which defined the in vivo AP population. The identity of these APs has been verified by their ability to differentiate in vitro and to reconstitute a functional fat pad in a lipodystrophic mouse, resulting in normalization of its blood glucose. Using flow cytometry, these APs are defined as CD45\(^{-}\); CD31\(^{-}\); CD29\(^{+}\); CD34\(^{+}\); Sca1\(^{+}\), with CD24 expression differentiating between subpopulations varying in commitment to the adipocyte lineage.

RNA-seq has become a valuable tool to identify alternative markers for APs and to identify subpopulations with varying functions. For example, Merrick et al. identified a DPP4\(^{+}\) population which progresses to the more committed ICAM1\(^{+}\) and CD142\(^{+}\) cells. RNA-seq was also used to identify a population defined by expression of LY6C and PDGFR\(\beta\), known as fibro-inflammatory progenitors, or FIPs. FIPs are non-adipogenic and inhibit the adipogenesis of other AP subpopulations. The existence of a non-adipogenic subpopulation of APs is further supported by Marcelin et al. who identified CD9 as a marker of a highly proliferative population of APs that promote fibrosis rather than adipogenesis.
Finally, Schwalie et al. used RNA-seq to identify the Areg population, a CD142\(^{+}\)
subpopulation of APs which inhibits adipogenesis in a paracrine mechanism\textsuperscript{69}. These subpopulations indicate that APs are a diverse cell type with varying functions. Further work will be needed to ascertain the role of these subpopulations in WAT homeostasis.

AP function also depends on adipose depot and microenvironment. For example, APs isolated from SWAT have greater adipogenic capacity \textit{in vitro} than do APs isolated from VWAT\textsuperscript{70}. In addition, SWAT and VWAT APs differ in their proliferative response to HFD. In male mice, VWAT APs undergo a transient burst of proliferation at the onset of HFD feeding which then subsides to baseline levels over the course of a week. Eight weeks later, this proliferative burst contributes to the formation of new adipocytes. In contrast, SWAT APs in males do not proliferate in response to HFD and do not result in hyperplasia\textsuperscript{19}. This depot specificity is not found in female mice which exhibit an adipogenic response to HFD in both SWAT and VWAT\textsuperscript{71}; however, male mice will primarily be used in the studies presented here. Diet-induced hyperplasia was determined to be dependent on Akt2-signaling, in a mechanism that is specific to obesity and HFD consumption and distinct from the adipose tissue establishment during development. These findings illustrate the importance of studying adipose hyperplasia \textit{in vivo} within the appropriate context. These findings also illustrate the need to identify the adipogenic stimulus in HFD consumption which this work will address.
**Fatty acid signaling and receptors**

When HFD is ingested, the lipids are packaged into chylomicrons for transport to the various tissues. In adipose tissue, membrane proteins facilitate the uptake of TGs into the tissue. For example, FATP1 is translocated to the plasma membrane by insulin, where it increases lipid uptake from the plasma\textsuperscript{32}. In addition to FATP1, CD36 is another lipid transporter, with fatty acid uptake decreasing by 60-70\% in CD30-ko mice\textsuperscript{72}.

In addition to storing dietary lipids, adipocytes and APs respond to them as signaling ligands. Dietary fats have been identified as a primary driver of adiposity, making them a potential candidate for AP activation and proliferation\textsuperscript{73}. Fatty acids and other lipid-derived compounds known as lipokines are important modifiers of metabolic health, able to improve insulin signaling and hepatosteatosis\textsuperscript{74}. Furthermore, fatty acids have also been shown to affect DNA methylation and adipokine secretion in adipocytes\textsuperscript{75,76}. Given the association with obesity and HFD, additional work is needed to understanding how dietary fatty acids impact adipose expansion and overall health.

The signaling functions of fatty acids in adipose tissue has spurred interest in their mechanisms. Fatty acids may signal via the Free Fatty Acid Receptor (FFAR) family, a group of G-protein coupled receptors which are categorized by the length of fatty acid to which they bind. This work will discuss Gpr120 (also known as FFAR4) and Gpr40 (also known as FFAR1) which bind to medium- and long-chain fatty acids\textsuperscript{77,78}. Gpr40 is primarily expressed in the pancreas and
intestines\textsuperscript{79} and is known to mediate the effects of fatty acids on glucose-stimulated insulin secretion, although whether it is harmful or beneficial has been controversial\textsuperscript{80}. Nagasumi et al. overexpressed Gpr40 in murine pancreatic beta cells and determined that overexpression improved glucose tolerance and glucose-stimulated insulin secretion in diet-induced obese mice\textsuperscript{81}. However, Steneberg et al. found that Gpr40\textsuperscript{+/} mice were protected from obesity-associated glucose intolerance and insulin resistance and that overexpressing Gpr40 under the pancreatic promoter PDX1 increased hepatic steatosis and decreased glucose tolerance and glucose-stimulated insulin secretion\textsuperscript{82}. Alquier et al. have attempted to reconcile these opposing views by noting that the PDX1 promoter expression is not restricted to the pancreas and is also expressed in the hippocampus. They also note that Steneberg et al.’s transgenic line may have suffered from developmental effects, as islet morphology appeared to be affected\textsuperscript{80}. They suggest that evidence points toward Gpr40 signaling as beneficial for metabolic health.

Unlike Gpr40, Gpr120 is known to be expressed in white adipose tissue. Furthermore, APs with reduced expression of Gpr120 have decreased adipogenic potential, with less lipid accumulation and decreased expression of the adipogenic genes Ppary-2 and aP2\textsuperscript{83}. Hilgendorf et al. supported the importance of Gpr120 in adipogenesis, observing that Gpr120 are localized on AP cilia\textsuperscript{84}. Although Gpr120\textsuperscript{+/} mice consuming a long-term HFD accumulate more adipose compared to their wild-type (WT) counterparts, embryonic fibroblasts derived from this mouse model have reduced adipogenic potential \textit{in vitro}. 
Adipocytes of these knockout obese mice are larger than WT, suggesting that they grow preferentially by hypertrophy rather than hyperplasia in response to diet\textsuperscript{85}.

In addition to its effects on adipogenesis, Gpr120 has been studied for its effects on inflammation and insulin resistance. Oh et al. observed that pharmacological activation of Gpr120 reduced inflammatory gene expression in an LPS-treated macrophage cell line, an effect that was inhibited when Gpr120 was knocked down. In addition, while supplementing a HFD with omega-3 fatty acids improved insulin sensitivity, omega-3 fatty acids had no effect on insulin sensitivity in Gpr120\textsuperscript{-/-} mice\textsuperscript{86}.

Another signaling receptor capable of responding to fatty acids is the liver X receptor (LXR)\textsuperscript{87}. This is a nuclear hormone receptor with two isoforms, α and β. While LXRβ is expressed in most tissues, LXRα is expressed in tissues with high lipid metabolism, such as brown and white adipose tissue, intestine, kidney, and liver\textsuperscript{88}. Published work on the role of LXRα in adipogenesis is contradictory. Seo et al. demonstrated increased adipogenesis in 3T3-L1 cells when treated with the LXRα activator, T0901317. Furthermore, they identified LXR binding sites in the promoter region of the adipogenic “master regulator” Pparγ. In contrast, Hummasti et al. did not observe any effects on 3T3-L1 differentiation when treated with another LXRα agonist, GW3965\textsuperscript{89}. This difference in results could be due to these agonists having different efficacies, with GW3965 being a weaker agonist in some cell types\textsuperscript{90}. 
While most published work has focused on LXR signaling via its ligand binding mechanism, LXRα modulation is complex and can be regulated by its posttranslational modifications. Torra et al demonstrated that LXRα activity differed if it was activated via ligand binding or via dephosphorylation, with the modulator T0901317 activating both these mechanisms\textsuperscript{91}. A later study used a mouse model containing a point mutation to induce a serine-to-alanine conversion at residue 196 in LXRα, eliminating the phosphorylation site in a macrophage cell line. This mutation resulted in a shift toward expression of proliferative genes in murine bone-derived macrophages. This shift in gene expression was determined to be independent of ligand binding by the LXRα agonist GW3965\textsuperscript{92}.

This work will elucidate mechanisms of adipogenesis, including investigating whether modulation of Gpr120, Gpr40, and/or LXRα plays a role in AP activation by diet. This work also leverages an isocaloric HFD screen with various fat sources, ultimately leading to the identification of a novel mechanism of AP proliferation, the characterization of the effects of dietary fat type on WAT gene expression and function, and greater context when considering WAT inflammation and fibrosis during obesity. This work aims to further our understanding of how dietary fats impact obesity while providing a quantitative assessment of parameters related to mechanisms of adipose expansion.
Chapter 2: Dietary oleic acid induces AP proliferation via down-regulation and dephosphorylation of LXRα

Results

Effects of dietary fats on adipose hypertrophy and hyperplasia

Jeffery et al. determined that adipose hyperplasia induced by HFD is a distinct process from hyperplasia to establish the WAT during development. However, the mechanism of diet-induced obesity remained unknown. To determine whether diet activation of APs is due solely to increased caloric intake, AP proliferation was quantified in mice pair-fed HFD to calorically matched mice fed a standard diet (SD) for 1 week.
Figure 1: Dietary fat source determines whether VWAT expands primarily via hypertrophy or hyperplasia. A) VWAT AP proliferation in mice fed SD or pair-fed HFD mice for 7 days. (n=5; unpaired t-test; experiment conducted by Christopher Church) B) AP proliferation in SWAT and VWAT normalized to the SD-fed VWAT proliferation. Mice were fed the indicated HFD for 7 days. (n=5-2; experiment conducted by Jennifer Kaplan) C) VWAT weights of mice which consumed the indicated HFD for 12 weeks.
(n=4-5; measured by Jennifer Kaplan) **D)** Quantification of newly formed adipocytes from AdiCre-ER/mTmG mice. Mice were injected with tamoxifen then given indicated HFD for 8 weeks. (n=3-18) **E)** Quantification of VWAT BrdU-labeled adipocyte nuclei. Mice were given indicated HFD with BrdU water for one week. BrdU water was then removed, and mice continued to consume indicated diet for a total of 12 weeks. (n=4-5). **F)** Mean VWAT adipocyte volume from mice which consumed the indicated HFD for 12 weeks. (n=4-5). Correlations between VWAT mass and **G)** average VWAT adipocyte diameter for each diet and **H)** average rate of adipocyte formation for each diet. (Significance calculated by one-way ANOVA multiple comparisons to SD or Spearman correlation)

Although both sets of mice consumed the same number of calories, VWAT APs of HFD-fed mice had increased proliferation compared to APs of SD-fed mice (Figure 1A). These results indicate that AP activation by diet is not due solely to increased caloric intake, suggesting that composition of the diet itself drives AP proliferation.

Increased dietary fat has been found to be more strongly associated with obesity compared to dietary carbohydrate and protein content. To determine if dietary fat sources impact adipogenesis, we designed a series of identical, isocaloric 45% fat diets with varying fat sources. Mice were fed these diets for one week along with drinking water containing BrdU to quantify AP proliferation. After one week, VWAT APs demonstrated a highly varied response to the HFDs, with some diets such as Fish and Palm inducing no significant change in AP proliferation while Soybean and Peanut resulted in an increased proliferation (Figure 1B). SWAT AP proliferation was not significantly increased compared to SD. These data indicate that dietary fat source and composition are highly relevant to AP activation and proliferation.

I next wanted to characterize the effects of these dietary fats on WAT expansion. WT mice that consumed the diets for 12 weeks demonstrated
variations in VWAT mass, with most diets increasing VWAT mass to varying degrees while other diets such as High Oleic (HO) Sunflower and HO Safflower did not increase VWAT compared to SD (Figure 1C). To determine whether diets that induced AP proliferation also resulted in increased adipocyte formation, diets were fed to the AdiponectinCre-ER/mTmG (AdCre-ER/mTmG) mouse model for 8 weeks to quantify adipogenesis as presented previously94. In brief, this model utilizes an inducible Cre response element regulated by the adiponectin promoter so that upon induction, adipocytes transition from expressing a red fluorescent reporter to a green one. When mice are fed the indicated HFD, any adipocytes that form after induction will be red, allowing for the quantification of in vivo adipogenesis. In addition to increased AP proliferation, the Lard, Olive, and Soybean diets resulted in increased adipocyte formation compared to SD (Figure 1D). However, HO Sunflower, HO Safflower, and Peanut diets which resulted in the most extreme increase in AP proliferation, resulted in lower rates of adipogenesis than expected and were not significantly increased. As an alternative measure of adipogenesis, I performed a BrdU pulse-chase to quantify BrdU-labeled adipocytes formed from diet-activated APs. In brief, mice were given the HFD and BrdU drinking water for one week. The BrdU was then removed, and the mice continued to consume the diet for 11 more weeks, allowing any activated APs to differentiate and form mature adipocytes. In this case, none of the diets resulted in a significant increase above SD within the context of the diet series (Figure 1E). However, these data appeared to mimic trends from the AdCre-ER/mTmG mice with Lard, Olive, and Soybean having
more BrdU-labeled nuclei and HO Sunflower, HO Safflower, and Peanut having fewer labeled nuclei. Therefore, in addition to driving different rates of AP proliferation, dietary fat source affects adipocyte formation as well.

Adipocyte size was quantified by staining fixed sections of adipose tissue with Masson’s trichrome stain and systemically imaging and quantifying adipocyte area. Interestingly, upon quantifying VWAT adipocyte volume, relatively few diets resulted in increased adipocyte size compared to SD. Yet diets with low adipocyte formation such as Butter and Palm diets had the most dramatic increases in adipocyte size. In contrast, diets resulting in greater adipocyte formation such as Olive and Soybean did not significantly increase adipocyte size (Figure 1F). These data suggest that dietary fat source may bias the tissue toward expanding primarily via hypertrophy or hyperplasia.

In previously published work, comparing fat mass gain during consumption of a 60% lard HFD to SD consumption illustrated that both adipose hypertrophy and hyperplasia contribute to overall WAT mass\textsuperscript{71,95}. However, we have used a series of HFDs, resulting in a variety of graded responses in fat mass gain. This approach allows us to separate and distinguish between these mechanisms to assess their effects on overall adipose mass. Quantifications of adipogenesis and adipocyte diameter from the dietary fat screen were correlated to each individual mouse’s VWAT mass. While adipocyte diameter had a strong and significant correlation to VWAT mass, adipogenesis demonstrated no such correlation, suggesting that in the more complex context of the dietary fat screen,
adipocyte size is a stronger driver of depot size than is adipogenesis (Figure 1G-H).

Figure 2: SWAT lacks an adipogenic response to HFDs and expands primarily via hypertrophy. A) SWAT mass after 12 weeks of diet consumption. (n=5; measured by Jennifer Kaplan) B) Quantification of newly formed SWAT adipocytes in AdiCre-ER/mTmG mice after 8 weeks of HFD consumption. (n=3-18) C) Quantification of proportion of BrdU-labeled SWAT adipocyte nuclei in mice which ate diet for 12 weeks. (n=4-5) D) Mean SWAT adipocyte volume after 12 weeks of consuming indicated HFD. (n=4-5). Correlations between SWAT mass and E) SWAT adipocyte diameter and F) SWAT adipocyte formation in AdiCre-ER/mTmG mice. (Significance calculated by one-way ANOVA multiple comparisons to SD or Spearman correlation)
In characterizing the WT SWAT mass after long-term consumption of indicated HFD, this tissue demonstrated a range of responses, with some strong increases in SWAT weight in diets such as Palm and Peanut and some moderate increases, such as during Palm consumption (Figure 2: **SWAT lacks an adipogenic response to HFDs and expands primarily via hypertrophy.** A) SWAT mass after 12 weeks of diet consumption. (n=5; measured by Jennifer Kaplan) B) Quantification of newly formed SWAT adipocytes in AdiCre-ER/mTmG mice after 8 weeks of HFD consumption. (n=3-18) C) Quantification of proportion of BrdU-labeled SWAT adipocyte nuclei in mice which ate diet for 12 weeks. (n=4-5) D) Mean SWAT adipocyte volume after 12 weeks of consuming indicated HFD. (n=4-5). Correlations between SWAT mass and E) SWAT adipocyte diameter and F) SWAT adipocyte formation in AdiCre-ER/mTmG mice. (Significance calculated by one-way ANOVA multiple comparisons to SD or Spearman correlation)A). In contrast to VWAT, SWAT adipocyte formation in the AdiCre-ER/mTmG mice was not significantly increased compared to SD on the various HFDs (Figure 2B). Additionally, although there was variation in the proportion of BrdU-labeled SWAT adipocyte nuclei, none of the diets resulted in significantly greater adipogenesis compared to SD by this measure of hyperplasia (Figure 2C). Finally, like VWAT, 12 weeks of consuming the various HFDs resulted in varying SWAT adipocyte sizes with the Lard and Butter diets inducing significantly increased adipocyte size compared to SD (Figure 2D). Also, similarly to VWAT, SWAT mass was significantly correlated to adipocyte size rather than adipocyte formation (Figure 2E-F). These data indicate that while there are depot-specific differences in how
dietary fats activate mechanisms of WAT expansion, hypertrophy remains the primary overall driver.

*Dietary oleic acid promotes AP proliferation and adipogenesis*

**Figure 3: Dietary oleic acid drives AP proliferation and differentiation.**

A) Correlation between dietary oleic acid content and average fold increase in VWAT AP proliferation after one week of diet consumption. (Spearman correlation) B) Total plasma fatty acid concentration during first week of 60% lard HFD consumption. (n=5; unpaired t-test) C) Percentage change in plasma oleic acid concentration compared to Day 0 of HFD consumption. (n=5; unpaired t-test) D) Oleic acid content in VWAT during HFD feeding. (n=3, unpaired t-test) E) Fat pad mass of mice which consumed SD with vehicle or SD supplemented with tri-oleate to 45% for 8 weeks (n=5; one-way ANOVA) F) AP
proliferation in SWAT and VWAT after one week of SD supplemented with vehicle or trioleate. (n=5; one-way ANOVA) G) VWAT AP proliferation after 5 days of SD supplemented with purified fatty acids. (n=5; one-way ANOVA) H) AP proliferation after daily jugular infusions of oleic acid (20 mM) or vehicle (BSA) for 5 days. (n=5; one-way ANOVA) I) Lipid accumulation quantified by Oil Red O staining after 7 days of differentiation of primary APs. APs differentiated with insulin, insulin supplemented with indicated fatty acid (100 µM), or MDI. (n=3; one-way ANOVA compared to insulin) J) Lipid accumulation in primary human APs differentiated with MDI or MDI supplemented with oleic acid (100 µM) for 7 days. Human APs defined as CD45−; CD235−; CD90+, GP38+, and CD34+. (n=3; unpaired t-test) (All experiments conducted by Christopher Church)

To determine mechanistically how dietary fats drive mechanisms of adipose expansion, lipidomic analysis was performed to determine the fatty acid composition of each diet. In correlating fatty acid composition to measures of hypertrophy and hyperplasia, oleic acid was found to be significantly and strongly correlated to AP proliferation (Figure 3A). This relationship contrasted with other fatty acids, such as 16:0, 18:0, and 18:2n-6, which are similar in structure and demonstrated no such correlation (data not shown). Previous studies have shown that AP proliferation peaks at Day 3 of HFD and subsides back to basal proliferation after the first week19. In quantifying plasma and adipose fatty acids after 3 or 7 days of a 60% lard-based HFD, total plasma fatty acids peaked at Day 3 and were decreased on Day 7 (Figure 3B). Plasma oleic acid mimicked the plasma fatty acid levels, increasing on Day 3 and decreasing on Day 7 (Figure 3C). In contrast, VWAT oleic acid increased steadily over the course of the week (Figure 3D). These results suggest that the decrease in plasma oleic acid and fatty acids is due to the absorption by WAT. These findings identify oleic acid as a potential nutritional signal driving *in vivo* AP proliferation.

To determine whether oleic acid induces AP proliferation or is merely correlated with it, SD was supplemented with tri-oleate, a more stable form of
oleic acid. These diets were supplemented to 45% kcal fat, matching the custom diet series. Long-term consumption of this supplemented diet was sufficient to increase VWAT mass, while there was no effect on SWAT mass (Figure 3E). When mice were fed these diets for one week, the addition of tri-oleate was sufficient to induce a significant increase in AP proliferation in the VWAT, while there was no effect in the SWAT (Figure 3F). As another approach to this experiment, stearic acid (18:0), oleic acid (18:1), and linoleic acid (18:2) were directly supplemented into a SD. Of these fatty acids, only oleic acid induced AP proliferation in the VWAT, while stearic and linoleic acids were no difference than SD (Figure 3G). To determine whether oleic acid itself or a metabolite formed during absorption is responsible for inducing AP proliferation, mice were given jugular catheters to allow oleic acid to be administered directly into the bloodstream. In this scenario, oleic acid was still sufficient to increase AP proliferation in VWAT (Figure 3H). These data show that dietary oleic acid is sufficient to induce AP proliferation in vivo.

To further characterize the specificity of oleic acid in driving adipose hyperplasia, primary APs were harvested and cultured in vitro. The cells were then differentiated using either insulin, an adipogenic cocktail of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (MDI), or insulin supplemented with a screen of fatty acids. Of the fatty acid screen, only oleic acid (C18:1) and palmitoleic acid (C16:1) resulted in significantly more lipid accumulation (Figure 3I). We also observed from our lipidomic data that the concentration of fatty acid used was physiologically relevant for oleic acid but was hyper-physiologic for
palmitoleic acid, suggesting that oleic acid remains a highly specific driver of adipogenesis. Finally, to determine whether oleic acid also increased lipid accumulation in human APs, we isolated primary APs from human adipose samples and differentiated them in the presence of oleic acid. Oleic acid significantly increased lipid accumulation (Figure 3J), suggesting this effect is not restricted to mice. These data show that oleic acid is capable of inducing adipogenesis in addition to AP proliferation.
Figure 4: OA increases adipogenesis by inducing Akt2 activation and lipogenesis.
A) Lipid accumulation in primary APs sorted from WT or Akt2−/− mice differentiated with
insulin, MDI, or insulin supplemented with oleic acid (18:1; 100 µM) for 7 days. (n=3; one-way ANOVA) B) Immunoblot and quantification of Akt2 phosphorylation at Serine 473 (S473) in primary APs after 3 hours of differentiation with insulin or insulin supplemented with oleic acid (18:1; 100 µM). (unpaired t-test) Gene expression of C) Pparγ, D) Cebpα, E) adiponectin, F) leptin, G) Fabp4, and H) perilipin in primary WT or Akt2−/− APs after 12 hours of differentiation with insulin or insulin supplemented with oleic acid (n=3; one-way ANOVA) I) Lipid accumulation in primary APs after 24 hours of differentiation with insulin, or insulin and oleic acid (OA; 100µM) with glucose uptake inhibitor or fatty acid synthase inhibitor. (± 25 mM glucose as indicated; n=3; unpaired t-test) (All experiments performed by Christopher Church)

Jeffery et al. found that Akt2 signaling is required for diet-induced adipogenesis yet is not required for hyperplasia during adipose establishment and development. To determine whether the ability of oleic acid to induce AP differentiation is dependent on Akt2, lipid accumulation was quantified in Akt2−/− APs. While oleic acid significantly increased lipid accumulation in the WT cells, this increase was ablated in the Akt2−/− cells (Figure 4A). To quantify Akt2 activation during oleic acid treatment, phosphorylation of residue S473 was quantified. After 3 hours of differentiation, oleic acid supplementation resulted in significantly more Akt2 activation than insulin alone (Figure 4B). In addition to lipid filling, Akt2 is also required for the characteristic changes in gene expression that accompany adipogenesis. While WT APs increased expression of Pparγ, Cebpα, adiponectin, leptin, Fabp4, and perilipin in response to oleic acid, Atk2−/− APs failed to increase expression of these genes during differentiation (Figure 4C-H). These data indicate that oleic acid acts in a signaling mechanism to activate Akt2 and suggests that oleic acid is a dietary ligand that promotes adipogenesis.

*AP proliferation is mediated by LXRα signaling*
Figure 5: Oleic acid decreases LXRα activity and phosphorylation. 

A) Heat map showing pathways which are up- or downregulated in sorted APs in response to 3 days of 60% lard HFD consumption (n=3-5). VWAT expression of 

B) LXRα and C) Srebp-1c after 3 days of SD or HFD consumption. (n=5) D) LXR activity quantified by a luciferase reporter assay in 3T3-L1s after 24 hours treatment with MDI, MDI with oleic acid (OA; 100 µM), or MDI with T0901317 (5 µM). (n=6) E) Dephosphorylation of LXRα is associated with upregulation of genes involved in proliferation. F) Western blot and quantification of p-LXRα in primary APs differentiated for 24 hours with insulin or insulin supplemented with oleic acid (OA; 100 µM), linoleic acid (LA; 100 µM), or T0901317 (5 µM). (n=1) (Significance calculated by unpaired t-test)
To identify a mechanistic pathway for how dietary oleic acid induces AP proliferation, I used our understanding of sex-dependent differences in WAT patterning. While male mice undergo diet-induced adipogenesis in the VWAT\textsuperscript{96}, HFD induces adipogenesis in the SWAT and VWAT of female mice (Appendix Figure 18). I used an RNA-seq dataset collected from the SWAT and VWAT APs of male and female mice fed either SD or 60% lard HFD for 3 days, the time of maximal diet-induced AP proliferation\textsuperscript{96}. I analyzed this dataset for pathways modulated by HFD that were consistent between the adipogenic depots—male VWAT and female SWAT and VWAT—and lacking in the nonadipogenic depot—male SWAT. Downregulation of the LXR/RXR pathway was identified to be a strong hit fulfilling these criteria (Figure 5A). Of the LXR isoforms, LXR\textalpha was of particularly interest given its role in activating genes involved in lipogenesis and cholesterol transport\textsuperscript{97}. LXR\textalpha is also known to be involved in oleic acid signaling, modulating oleic acid-induced hepatic lipogenesis\textsuperscript{98}, and protecting against oleic acid-induced pulmonary inflammation\textsuperscript{99}.

To verify the results of the RNA-seq pathway analysis, APs were sorted from SWAT and VWAT of male mice fed either SD or 60% HFD for 3 days. Gene expression of LXR\textalpha and other genes downstream of LXR\textalpha signaling was then quantified. Interestingly, there was no change in expression of LXR\textalpha itself (Figure 5B). However, expression of Srebp-1c, a gene regulated by LXR\textalpha signaling was decreased in the HFD SWAT and trending toward decreased expression in the VWAT (Figure 5C). These data suggest that while LXR\textalpha expression was not
affected, at least one of its downstream signaling pathways was downregulated, consistent with the RNA-seq dataset.

To determine whether LXRα activity was being modulated specifically by oleic acid, 3T3-L1s were transfected with a luciferase reporter to quantify LXR activity. Cells were also treated with the LXRα modulator T0901317 as a positive control. While the T0901317 resulted in a strong increase in LXR activity, supplementation of oleic acid in the adipogenic cocktail significantly decreased LXR activity relative to MDI alone (Figure 5D). As LXRα expression was not affected by short-term HFD consumption, we considered posttranslational modifications as an alternative mechanism of regulation. The modulator T0901317 has been shown to increase LXRα phosphorylation\textsuperscript{91}. Furthermore, dephosphorylation of LXRα has been found to promote expression of proliferative genes in bone-derived macrophages (Figure 5E)\textsuperscript{92}. The mechanisms of this signaling are still being understood. To determine whether oleic acid affects LXRα phosphorylation, primary APs were cultured and differentiated for 24 hours. Treatment with oleic acid slightly decreased LXRα phosphorylation, while treatment with linoleic acid and T0901317 resulted in increased LXRα phosphorylation (Figure 5F). While this experiment will be repeated in the near future, these data suggest that oleic acid decreases LXRα activity and its phosphorylation, which could increase proliferation of the cells.
Figure 6: LXRα phosphorylation is inhibitory to VWAT diet-induced AP proliferation. A) AP proliferation in mice given 60% Lard HFD and daily injections of vehicle (MCT) or T0901317 (50 mg/kg) for 3 days with BrdU drinking water. (n=5) B) Quantification of the percentage of APs (Sca1+ cell) which are p-LXRα+. (n=4-5) C) Quantification of VWAT and D) SWAT AP proliferation measured by Ki67 expression in WT (C5Bl/6J) or S196A LXRα mice after 3 days of SD or HFD consumption. (n=5-6) (Significance measured by unpaired t-test)

To quantify how modulation of LXRα signaling affects AP proliferation in vivo, mice were treated with T0901317 along with 3 days of 60% lard HFD
feeding. While T0901317 did not affect AP proliferation relative to vehicle in the SWAT over this time course, it led to a decrease in VWAT AP proliferation (Figure 6A). This depot specificity suggests that LXRα activation prevents diet-induced AP proliferation.

To determine how LXRα phosphorylation is affected by short-term HFD consumption in vivo, immunohistochemistry was used to stain VWAT with Sca1 to mark APs and p-LXRα. The proportion of APs with phosphorylated LXRα was then quantified in mice fed SD or HFD for 3 days. Short-term HFD consumption resulted in decreased APs with phosphorylated LXRα (Figure 6B). Finally, to determine whether decreased LXRα phosphorylation is involved in diet-induced AP proliferation, mice with a serine-to-alanine mutation at an LXRα phosphorylation site (S196A) were obtained. These mice were fed SD or 60% lard HFD for 3 days. VWAT AP proliferation in the S196A mice was not affected when the mice consumed SD. However, when the mice were given HFD, VWAT AP proliferation in the S196A mice was strongly induced and was significantly greater than AP proliferation in the HFD-fed WT mice (Figure 6C). This finding indicates that LXRα phosphorylation is inhibitory to diet-induced AP proliferation but does not affect basal VWAT proliferation. Interestingly, in the SWAT, while there was no diet-induced AP activation, proliferation in the SD- and HFD-fed S196 mice were significantly increased over the WT proliferation, suggesting that LXRα phosphorylation may be also be involved in inhibiting the proliferative response in SWAT (Figure 6D). These data show that LXRα phosphorylation inhibits diet-induced AP proliferation.
Discussion

Using a matched series of HFDs, we have shown that the type of dietary fat influences whether adipose expands primarily through hyperplastic or hypertrophic mechanisms, or both. This HFD screen identifies oleic acid as a dietary driver of AP proliferation that increases Akt2 phosphorylation and lipogenesis. Finally, I have also identified LXRα phosphorylation as a inhibitory mechanism of diet-induced AP proliferation.

Many other studies have illustrated the significant role fatty acids play in regulating adipose phenotypes, such as inflammation \textsuperscript{100}, adiposity \textsuperscript{101}, and insulin signaling \textsuperscript{102}. However, we utilized a series of isocaloric HFDs that are identical except for the fat sources, allowing us to focus on the \textit{in vivo} effects of dietary fats without being confounded by differing carbohydrates and proteins between the diets. Furthermore, the use of several HFDs, as opposed to comparing a low-fat diet to HFD, has yielded more graded responses, allowing the identification of trends that may otherwise be obscured by studying two extremes in fat content. For example, while we have previously shown that adipogenesis correlates with adipose depot weight when comparing SD to a 60% HFD \textsuperscript{103}, our approach allowed us to define a more nuanced context of many HFDs in which adipocyte size was found to be more tightly linked to depot weight.

Using correlations between dietary fatty acid composition and measures of adipose expansion, oleic acid was identified as a dietary signal inducing AP activation and proliferation. The primary source of oleic acid in the human diet is
olive oil, which is a major component of the Mediterranean diet. In addition to its association to decreased cardiovascular disease and cancer risks \(^{104,105}\), the Mediterranean diet has also been studied as a potential therapy for weight loss\(^{106,107}\). In other studies, it has been praised as a palatable, non-obesogenic diet, despite its high proportion of fat, primarily comprising of olive oil \(^{108–110}\). Therefore, it is perhaps surprising that our findings identify oleic acid as a dietary driver of AP proliferation. However, Mediterranean diet is characterized by several other parameters, including high consumption of fruits, vegetables, legumes, and unrefined cereals and grains which can also have benefits on health \(^{111}\). Additionally, although oleic acid is a major component of olive oil, extra virgin olive oil, a healthier variation of refined olive oil, also contains many other compounds such as polyphenols and vitamins. In particular, polyphenols have been identified as the potential drivers of the cardiovascular and inflammatory benefits of olive oil \(^{112–114}\). Therefore, this identification of oleic acid as a dietary signal inducing AP activation is not necessarily inconsistent with the Mediterranean diet’s association with longevity.

Furthermore, lipodystrophic models illustrate the dire consequences to metabolic health in the absence of adipose, indicating that some adipose tissue is necessary to store and manage lipids \(^{115,116}\). This metabolic phenotype can even be reversed with the addition or reinstallation of white adipose tissue \(^{64,117}\). The overflow model expands upon this idea, identifying adipose tissue expansion as a compensatory mechanism to sequester lipids away from ectopic storage such as in muscle and liver \(^{118–120}\). Furthermore, the formation of many, small
adipocytes has been related to improved insulin sensitivity and metabolic health. Adipogenesis is also a hypothesized mechanism behind thiazolidinediones, a class of Ppar-γ agonists that improve insulin resistance, but are associated with adipose gain through adipogenesis. Further work on mice consuming this HFD series will be able to reveal whether dietary oleic acid is associated with any metabolic pathologies.

Our data finds that oleic acid promotes adipogenesis via Akt2 signaling and stimulation of lipogenesis. These data also make the novel discovery of LXRα phosphorylation as a regulatory mechanism to inhibit AP proliferation. Knockout of LXRα in adipose tissue has been published to result in substantially increased fat mass and larger adipocyte size. Additionally, prolonged treatment of ob/ob mice with the LXRα agonist GW3965 resulted in redistribution of adipose tissue shifting from VWAT to SWAT but did not alter adipocyte size or number. However, while these mouse models target expression of LXRα or activate it via ligand binding, our hypothesis addresses modulation of LXRα signaling via its dephosphorylation. The mechanism of LXRα activation, whether through ligand or posttranslational modification, regulates its downstream activity and signaling. I observed high basal rates of LXRα phosphorylation in VWAT APs, consistent with the low basal rates of AP proliferation during SD consumption. Additionally, I observed increased VWAT AP proliferation during HFD consumption, but no effect on AP proliferation of SD-fed mice, suggesting that the S196A mutations affects diet-induced proliferation, rather than basal proliferation. These data identify a novel mechanism to regulate AP proliferation.
in response to HFD. Further work should be done to determine whether LXRα phosphorylation also plays a role in AP differentiation or if it is solely involved in AP activation.

Our HFD series has illustrated the significant influence dietary fats play in mechanisms of adipose expansion. We have also identified oleic acid as a dietary fatty acid capable of inducing AP proliferation and lipogenesis. Other fatty acids should be studied to determine whether they mediate the many other health disorders associated with obesity. I have identified LXRα phosphorylation as a novel regulator of AP activation. The ability of this HFD series to bias adipose tissue expansion toward hypertrophy or hyperplasia provides a tool to understand how these mechanisms relate to metabolic pathologies associated with obesity.
Chapter 3: Gpr120 and Gpr40 are not required for diet-induced AP proliferation

Results

Gpr120 and Gpr40 do not mediate diet-induced AP proliferation

With the identification of dietary oleic acid as a driver of AP proliferation, I was motivated to consider potential receptors that could mediate oleic acid signaling, which led me to focus on Gpr120 and Gpr40. These receptors belong to the Free Fatty Acid Receptor family and are activated by medium- to long-chain fatty acids\textsuperscript{77,127,128}. While Gpr40 has primarily been studied for its role in insulin-stimulated glucose secretion in the pancreas\textsuperscript{129}, Gpr120 is expressed in WAT, and has been shown to promote differentiation of 3T3-L1 cells\textsuperscript{83}. However, in vivo work on the role of Gpr120 in adipogenesis has been limited.

To determine the effects of Gpr120 and Gpr40 on adipose hyperplasia, we obtained whole-body Gpr120-knockout (Gpr120KO) and Gpr40-knockout (Gpr40KO) mice. To verify the knockout and ensure that knockout of one receptor did not result in a compensatory increase in expression of the other receptor, I quantified gene expression in these mice.

![Figure 7: Expression of Gpr40 and Gpr120 does not increase in the knockout of the other receptor. A) Expression of Gpr40 in sorted SWAT and VWAT APs from WT,](image-url)
Gpr120KO, and Gpr40KO mice. (n=3) B) Expression of Gpr120 in sorted SWAT and VWAT APs from WT, Gpr120KO, and Gpr40KO mice. (n=3; two-way ANOVA)

The Gpr40KO mice demonstrated highly efficient knockout of Gpr40 expression in both the SWAT and VWAT (Figure 7A). Expression of Gpr120 was also nearly eliminated in the Gpr120KO mice in both SWAT and VWAT (Figure 7B). Interestingly, Gpr40 expression was also decreased in the Gpr120KO VWAT. These data indicated that the knockout mouse models were highly efficient and did not result in compensatory overexpression of the opposite receptor.

Previous work in the lab quantified proliferating APs in whole-body Gpr120 or Gpr40 knockout mice. After 3 days of HFD consumption, while proliferation in VWAT of Gpr120KO APs was similar to that of WT mice, proliferation in VWAT Gpr40KO APs was decreased compared to WT mice. These data initially identified Gpr40 as a potential mediator of diet-induced AP proliferation.
Figure 8: Gpr40 and Gpr120 are not required for diet-induced AP proliferation. VWAT AP proliferation in WT and Gpr40KO mice fed HFD for 7 days. (n=4-16) B) VWAT
To repeat this result, I subjected Gpr120KO and Gpr40KO mice to our typical assay of one week of HFD\textsuperscript{19}. In contrast to previous results, in the VWAT, both Gpr120KO and Gpr40KO mice exhibited increased AP proliferation when HFD-fed compared to Chow-fed knockouts (Figure 8A-B). As expected for male mice, neither the Gpr40KO or Gpr120KO mice demonstrated diet-induced AP proliferation in the SWAT (Figure 8C-D). As these receptors bind to similar ligands, one hypothesis was that the two receptors may act redundantly. To address this idea, I bred mice that lacked both the Gpr120 and Gpr40 receptors. However, when these mice were fed a HFD for one week and compared to their heterozygous littermates, there was no still difference in VWAT AP proliferation (Figure 8E). Again, as expected, there was no difference in the SWAT AP proliferation in HFD-fed double-knockout mice compared to heterozygous littermates (Figure 8F). These data indicate that neither Gpr120 nor Gpr40 are required for diet-induced AP proliferation.

Gpr40 mediates secretion of intestinal hormones, such as cholecystokinin which aids in digestion of fats and proteins\textsuperscript{130}. This role in lipid digestion could impact the timing of fatty acid absorption and could contribute to the apparent discrepancies between previous data in which decreased AP proliferation in Gpr40KO mice was observed and my results that fail to detect any effect. To test
this hypothesis, I quantified AP proliferation during 0-3 days and 3-6 days of HFD consumption. There was no significant induction of AP proliferation during days 0-3 of HFD, explaining how our initial, 3 day experiment identified Gpr40 deletion as inhibitory to AP proliferation. However, during 3-6 days, AP proliferation was strongly induced in the Gpr40KO mice, even compared to WT mice, suggesting that AP proliferation is not inhibited but merely delayed in these mutant mice (Figure 8G).

To remove the effects of this delayed response, we sought to determine whether AP-intrinsic expression of Gpr40 impacted hyperplasia by performing cell transplants as detailed in Jeffery et al\textsuperscript{131}. In brief, Gpr40KO mice were crossed to mTmG mice, causing all cells to express the red fluorescent protein Tomato in their membranes. APs were harvested from these mice and injected into the VWAT of B6 mice, whose cells lacked the Tomato expression. In comparing the proliferation of the endogenous, WT cells and the donated, Gpr40KO cells, there was no difference in proliferation between either of these cell types (Figure 8H). These data demonstrate that AP expression of Gpr40 is not required for a proliferative response to HFD.

\textit{Gpr40 and Gpr120 do not affect adipose hypertrophy or hyperplasia}

While Figure 8 indicates that Gpr120 and Gpr40 were not required for a proliferative response to HFD, these receptors could nonetheless affect subsequent differentiation after the activation phase. To test this idea, I fed HFD to Gpr120KO and Gpr40KO mice for 8 weeks.
Figure 9: Knockout of Gpr40 and Gpr120 does not affect adipocyte hyperplasia or hypertrophy. A) Body weights over time of WT or Gpr40KO mice fed SD or HFD for a total of 8 weeks. (n=5-6) B) Body weights over time of WT or Gpr120KO mice fed SD or HFD for a total of 8 weeks. (n=4-5; multiple t-test comparing Gpr40KO HFD to WT HFD) C) Mass of VWAT from WT or Gpr40KO mice. D) Mass of VWAT from WT or Gpr120KO mice.
mice fed SD or HFD. E) Mass of SWAT from WT or Gpr40KO mice fed SD or HFD. F) Mass of SWAT from WT or Gpr40KO mice fed SD or HFD. G) Percentage of BrdU-labeled adipocyte nuclei in VWAT of WT or Gpr40KO mice fed HFD for 8 weeks. H) Percentage of BrdU-labeled adipocyte nuclei in VWAT of WT or Gpr120KO mice fed HFD for 8 weeks. I) Average adipocyte volume in VWAT of WT or Gpr40KO mice fed HFD for 8 weeks. J) Average adipocyte volume in VWAT of WT or Gpr120KO mice fed HFD for 8 weeks.

While the Gpr40KO mice were not heavier than the WT mice on both HFD and Chow (Figure 9A), the HFD-fed Gpr120KO mice were significantly heavier than the HFD-fed WT mice (Figure 9B). This difference becomes significant at 3 weeks of HFD consumption which is earlier than the 8 weeks needed to for activated APs to differentiate. Interestingly, while the HFD-fed Gpr40KO mice consumed similar quantities of food to the HFD-fed WT mice (Figure 9C), the HFD-fed Gpr120KO mice consumed more calories during the initial period of diet consumption (Figure 9D). When quantifying depot weight, while the VWAT of the Gpr120KO and Gpr40KO mice were similar to WT mice (Figure 9E-F), the SWAT of the HFD-fed Gpr120KO mice was heavier than the SWAT of the WT mice (Figure 9E-H). The SWAT of the HFD-fed Gpr40KO mice was not statistically increased from the SWAT of the WT mice. This characterization of these knockout mice indicates that while there are slight differences in the Gpr120KO body weights and SWAT, there is no difference in the mass of the adipogenic depot, the VWAT, in either of these knockouts.

To determine whether these receptors impact adipogenesis, I performed a BrdU pulse-chase as presented previously. In brief, mice were given BrdU drinking water along with HFD for one week after which the BrdU was removed, and the mice continued to consume HFD for a total of 8 weeks. This assay
allows quantification of APs that were activated to differentiate by the HFD. After 8 weeks of HFD, there was no difference in the proportions of BrdU-labeled adipocyte nuclei in either HFD-fed Gpr120KO or Gpr40KO mice compared to WT mice (Figure 9I-J). Finally, to determine whether knockout of Gpr120 or Gpr40 impacted adipose hypertrophy, I quantified adipocyte volume from confocal microscopy images taken of stained tissue. There was no statistically significant difference between the volumes of VWAT adipocytes in WT and Gpr120KO or Gpr40KO mice (Figure 9K-L), indicating that. These data indicate that Gpr120 and Gpr40 do not affect adipose hypertrophy or hyperplasia.

**Discussion**

Given the work identifying dietary oleic acid as a driver of AP proliferation, we sought to identify receptors that mediate this signaling. However, using a variety of assays, my studies clearly demonstrate that Gpr120 or Gpr40 do not exert strong influences on AP proliferation or differentiation.

Previous works have suggested that knockout of Gpr120 reduces adipogenesis\textsuperscript{132}, while treatment with small molecule Gpr120 agonists increase \textit{in vitro} adipogenesis\textsuperscript{133,134}. However, these studies have been \textit{in vitro}, using either primary cells or the adipocyte precursor cell line 3T3-L1. In contrast, in using a whole-body knockout of Gpr120, there was no impact on \textit{in vivo} AP proliferation or differentiation. Given that our mouse model was a whole-body knockout, perhaps a more targeted knockout only in APs would reiterate the
results observed *in vitro*. Nonetheless, our data demonstrate the need to verify *in vitro* findings *in vivo*.

Ichimura et al. identified that knockout of Gpr120 exacerbates diet-induced obesity, with Gpr120KO mice exhibiting increased fat mass and adipocyte area when consuming either SD or HFD\(^{132}\). While I also determined that HFD-fed Gpr120KO mice were heavier than their WT counterparts, I did not identify any impairment in adipocyte formation or size. However, obesity studies often last for longer than 8 weeks, and continuing to feed GPR120KO mice beyond the 8 weeks could recapitulate a significant increase in adipocyte size, as published.

Very little has been studied about the role of Gpr40 in adipogenesis as this receptor is not highly expressed in adipose tissue\(^ {128}\). My data establishes that APs do indeed express low levels of Gpr40. However, knockout of Gpr40 did not affect AP proliferation, differentiation or body weight. This finding was consistent even when Gpr40KO APs were transplanted into WT mice, indicating that my findings are not due to whole-body knockout of the receptor. Therefore, while Gpr40 signaling impacts other facets of metabolism, it is less relevant for obesity\(^ {135,136}\).

My data has demonstrated that the FFARs Gpr120 and Gpr40 are not required for an adipogenic response to HFD. However, these receptors play vital roles in systemic metabolism, and Gpr120 expression is demonstrated to be important to regulation of obesity. Further work will be needed to determine if this function of Gpr120 is due to its WAT-specific expression or due to expression in other tissues.
Chapter 4: HFD screen impacts WAT gene expression and identifies relationships between WAT expansion mechanisms and adipose function

Results

_Dietary fat screen identifies relationships between adipokine expression and mechanisms of adipose expansion_

Mechanisms of adipose tissue expansion have been linked to varying outcomes for tissue function and systemic metabolic health, with hyperplasia being linked to a more metabolically healthy WAT and hypertrophy being linked to inflammation, fibrosis, and systemic insulin resistance\textsuperscript{137}. I have demonstrated that dietary fat source is highly relevant in modulating expansion primarily via hypertrophy or hyperplasia. Furthermore, our HFDs induce varying responses in WAT gain, enabling us to study the effects of graded levels of obesity rather than comparing the extremes of lean or obese mice. These observations illustrate the potential of our 45% HFD screen as a tool to study how dietary fats affect adipose function and how these changes are related to mechanisms of adipose expansion.

To characterize how dietary fat source impacts expression of adipokines, qPCR was used to quantify gene expression of leptin and adiponectin in mice which had consumed the HFD series for 12 weeks.
Figure 10: VWAT adiponectin expression correlates negatively with hypertrophy and dietary and plasma palmitic acid. A) VWAT expression of adiponectin from mice fed indicated HFD for 12 weeks. Normalized to expression of β-actin. (n=5-10; one-way ANOVA compared to SD). Correlations between average VWAT adiponectin expression for each diet and B) average VWAT adipocyte diameter, C) average VWAT hyperplasia quantified using the AdiCre-ER/mTmG mice, D) average VWAT AP proliferation during the first week of HFD consumption, E) dietary palmitic acid content of each HFD, F) average plasma concentration of nonesterified palmitic acid after 12 weeks of HFD consumption, and G) plasma concentration of total palmitic acid after 12 weeks of HFD consumption. (significance assessed using Spearman correlation)

In the VWAT, while the Lard, Butter, and Palm diets significantly decreased adiponectin expression compared to SD, mice consuming the other diets displayed no change in expression compared to SD (Figure 10A). As adiponectin has been linked to adipocyte size, it is perhaps expected that expression of
adiponectin and measures of adipose tissue expansion were correlated. Consistent with other publications\textsuperscript{47,48,50}, VWAT adipocyte size was negatively correlated to VWAT adiponectin expression, while there was no such correlation between VWAT adiponectin expression and hyperplasia measures such as VWAT adipocyte formation and VWAT AP proliferation (Figure 10B-D). Interestingly, VWAT adiponectin expression was negatively correlated to palmitic acid content in the HFD, plasma palmitic acid in the form of non-esterified fatty acid, and total plasma palmitic acid in all forms (Figure 10E-G). These data indicate that dietary fat type impacts VWAT adiponectin expression, possibly via palmitic acid content of the diet.

![Graph showing expression of adiponectin in SWAT adipocytes.](image)

**Figure 11:** SWAT adiponectin expression does not correlate to measures of adipose expansion. A) SWAT expression of adiponectin from mice fed indicated HFD for 12 weeks. Normalized to β-actin expression (n=5-10; one-way ANOVA). Correlations between average SWAT adiponectin expression for each diet and B) average SWAT adipocyte diameter for each HFD, C) average SWAT hyperplasia quantified using the AdiCre-ER/mTmG mice, and D) average SWAT AP proliferation after HFD consumption for one week.
In contrast to the VWAT, SWAT expression of adiponectin was only increased in mice that ate the Soybean diet (Figure 11A). Additionally, there were no correlations between SWAT adiponectin expression and SWAT adipocyte size, formation, or SWAT AP proliferation (Figure 11B-D), suggesting that there is a depot specificity in adiponectin’s relationship to adipocyte hypertrophy and that SWAT adiponectin expression may be regulated through other mechanisms.

In addition to adiponectin, leptin expression was also quantified after 12 weeks of diet consumption.
Figure 12: VWAT expression of leptin is linked to hyperplasia, rather than hypertrophy. A) VWAT expression of leptin from mice fed indicated HFD for 12 weeks. Normalized to β-actin expression. (n=5; ** one-way ANOVA compared to SD; # unpaired t-test to SD) Correlations between average VWAT leptin expression for each diet and B) average VWAT adipocyte diameter after 12 weeks of diet consumption, C) average adipocyte formation quantified in AdiCre-ER/mTmG mice, and D) average percentage of BrdU-labeled VWAT adipocyte nuclei. Correlations between average plasma concentration of leptin after 12 weeks of HFD consumption and E) average VWAT adipocyte diameter, F) average VWAT adipocyte formation as quantified in AdiCre-ER/mTmG mice, and G) average VWAT BrdU-labeled adipocyte nuclei. F) Correlation
between average VWAT leptin expression and average VWAT proliferative APs from each HFD. (Significance of correlations measured using Spearman correlation) **G**

VWAT leptin expression in WT or PdgfRα-Cre/Akt2\(^{fl/fl}\) after consuming SD or HFD for 8 weeks. Normalized to expression of β-actin. (n=2-5; unpaired t-test) **H**

Distribution of adipocyte diameters from WT or PdgfRα-Cre/Akt2\(^{fl/fl}\) mice fed either SD or HFD for 8 weeks.

Mice that consumed Olive and Soybean diets had increased leptin expression compared to SD, although expression on the Lard diet would also be considered significant if this diet were compared directly to SD (Figure 12A). As Leptin expression has previously been linked to adipocyte size\(^{138,139}\), correlations were again performed between VWAT leptin expression and mechanisms of VWAT expansion. In assessing correlations to determine the relationship between leptin expression and hypertrophy and hyperplasia, VWAT leptin expression surprisingly bore no relationship to VWAT adipocyte size (Figure 12B). Instead, VWAT leptin expression correlated significantly with two measures of hyperplasia—VWAT adipocyte formation quantified from the AdiCre-ER/mTmG mouse model, and BrdU-labeled adipocyte nuclei (Figure 12C-D). To verify whether these relationships were limited to gene expression, correlations to quantification of plasma leptin were also analyzed. While there was no correlation between plasma leptin and VWAT adipocyte size, plasma leptin was significantly correlated to the proportion of BrdU-labeled adipocyte nuclei, while adipocyte formation approached, but did not achieve, significance (Figure 12E-G). These findings show that leptin production did not correlate to hypertrophy and surprisingly correlated to measures of hyperplasia.

In examining the relationship between VWAT leptin expression and AP activation, 9 out of the 12 HFDs demonstrated a very strong correlation to VWAT
leptin expression (Figure 12H). However, the other three diets, HO Sunflower, HO Safflower, and Peanut, fall away from this trend. Interestingly, these three diets all have high rates of AP proliferation, yet lower rates of adipocyte formation (Figure 1D), highlighting the relationship to adipocyte formation.

After observing a correlation between leptin expression and adipocyte formation, I wanted to examine further whether adipocyte formation causes an increase in tissue-level leptin expression or whether this relationship is merely correlational. Akt2−/− mice lack an adipogenic response to HFD, yet have normal rates of adipocyte formation during WAT development, making these mice an effective tool to study processes related to obesogenic hyperplasia. To target Akt2 knockout to APs, I used a PdgfRα-Cre/Akt2fl/fl mouse line which targets the APs to compare leptin expression. While HFD-fed WT mice had increased VWAT leptin expression compared to SD, HFD failed to increase leptin expression in the PdgfRα-Cre/Akt2fl/fl mice (Figure 12I). Furthermore, when quantifying adipocyte size in these mice, there was no difference in adipocyte hypertrophy in HFD-fed WT mice compared to HFD-fed PdgfRα-Cre/Akt2fl/fl mice, further demonstrating that leptin expression is not dependent on adipocyte size (Figure 12J). This experiment shows that increased leptin expression due to HFD consumption is ablated in mice that are lacking an adipogenic response to the diet.
Figure 13: SWAT leptin expression is not correlated to measures of adipose expansion. A) SWAT expression of leptin in mice which consumed indicated HFD for 12 weeks. Data are normalized to β-actin. (n=5; one-way ANOVA) Correlations between average SWAT leptin expression after 12 weeks of indicated HFD consumption and B) average SWAT adipocyte diameter, C) average SWAT adipocyte formation measured in AdiCre-ER/mTmG mice, D) average SWAT AP proliferation during first week of HFD consumption.

In contrast to VWAT, in the SWAT, many diets—Butter, Palm, Coconut, Soybean, and Peanut—resulted in increased leptin expression (Figure 13A). However, SWAT expression of leptin was not correlated to any measures of adipose expansion, including SWAT adipocyte diameter, SWAT adipocyte formation, or the percentage of BrdU-labeled SWAT adipocyte nuclei (Figure 13B-D), showing that SWAT leptin expression is not related to any mechanisms of adipose expansion.

Many dietary fats do not promote increased expression of inflammatory or fibrotic markers
Figure 14: HFD screen identifies correlation between Tnf-α and measures of hyperplasia. VWAT expression of A) Tnf-α, B) IL-6, and C) IL-1β from mice fed indicated HFD for 12 weeks. Gene expression is normalized to β-actin expression. (n=5, * one-way ANOVA multiple comparisons to SD, # unpaired t-test to SD) Correlations between average VWAT expression of Tnf-α for each diet and D) average VWAT adipocyte diameter for each diet, E) average percentage of BrdU-labeled VWAT adipocyte nuclei, and F) average proliferation of VWAT APs after one week of HFD consumption. (Spearman correlation)

In addition to modulation of adipokine production, obesity is linked to measures of systemic and WAT-resident inflammation\textsuperscript{140,141} and adipose fibrosis\textsuperscript{44,142}. Increased WAT expression of inflammatory and fibrotic genes is
linked to increased plasma insulin\textsuperscript{143} and decreased insulin sensitivity\textsuperscript{144,145}. To quantify markers of inflammation, I selected Tnf-\(\alpha\), IL-6, and IL-1\(\beta\) due to their published increases in expression during obesity and their association to worsened metabolic health\textsuperscript{143,145–147}. Interestingly, when quantifying Tnf-\(\alpha\) expression in VWAT, the Fish and Palm diets resulted in increased Tnf-\(\alpha\) expression (Figure 14A). For comparison, a 60\% Lard diet, which is classically used in obesity studies\textsuperscript{148} was also used, which resulted in increased Tnf-\(\alpha\) expression when compared directly to the Chow diet. Expression of IL-6 was also quantified, with only the 45\% Lard diet resulted in increased VWAT IL-6 expression (Figure 14B). Finally, VWAT IL-1\(\beta\) expression was quantified with Soybean diet resulting in increased expression (Figure 14C). Interestingly, both 45\% and 60\% Lard HFDs resulted in significantly lower expression of IL-1\(\beta\) when compared directly to SD. These results demonstrate the importance of dietary fat type to WAT gene expression, with some diets increasing expression of these inflammatory genes and others having no effect.

In assessing how expression of inflammatory factors correlate to mechanisms of adipose expansion, VWAT expression of Tnf-\(\alpha\) was negatively correlated to two measures of hyperplasia- proliferative APs and BrdU-labeled adipocyte nuclei (Figure 14D-E). In contrast, there was no correlation between Tnf-\(\alpha\) expression and VWAT hypertrophy (Figure 14F). However, expression of these inflammatory markers was not correlated to measures of individual fatty acids. This correlation exemplifies the utility of this HFD series which produces a
range of responses, allowing for correlational analyses across many intermediates.

**Figure 15: Butter HFD increases expression of some inflammatory markers in SWAT.** Mean SWAT expression of A) Tnf-α, B) IL-6, and C) IL-1β from mice which consumed the indicated HFD for 12 weeks. Gene expression is normalized to expression of β-actin. (n=5; one-way ANOVA multiple comparisons to SD)

Gene expression of inflammatory cytokines was also characterized in SWAT. None of the diets resulted in a significant change in Tnf-α expression (Figure 15A). However, both Butter and Cocoa diets resulted in increased expression of IL-6 in the SWAT (Figure 15B). The Butter diet also resulted in significantly increased expression of IL-1β compared to SD (Figure
None of the SWAT expression of these inflammatory markers correlated significantly to measures of adipose expansion (data not shown).

In addition to inflammatory markers, Tgf-β1, Col1a1, Col3a1, and Col6 were selected as fibrotic genes which are strongly upregulated with obesity. VWAT expression of Tgf-β1 was only increased in mice consuming the 60% Lard diet. Interestingly, the 45% Lard diet resulted in the opposite change, with a slight but significant decrease in Tgf-β (Figure 16A). VWAT expression of Ctgf was significantly increased in mice which ate the Coconut diet (Figure 16B).

Figure 16: VWAT expression of fibrotic markers is primarily increased with consumption of 60% lard diet. VWAT expression of A) Tgf-β1, B) Ctgf, C) Col1a1, and D) Col6 in mice which had eaten one of HFDs for 12 weeks. Gene expression is normalized to expression of β-actin. (n=5, * one-way ANOVA multiple comparisons to SD, # unpaired t-test to SD)
Similar to VWAT Tgf-β1 expression, VWAT expression of Col1a1 was only significantly increased on the 60% Lard diet when compared directly to SD (Figure 16C). Finally, VWAT expression of Col6 was increased in the Safflower and HO Safflower diets, although it was also increased in both the 45% and 60% Lard diets when they were compared directly to SD (Figure 16D). These results show that while expression of several of these genes is increased in the context of the 60% lard HFD, the gene expression is not affected in many other diets which also induced obesity.

Figure 17: Dietary fat source has varying effects on SWAT expression of fibrotic markers. SWAT expression of A) Tgf-β1, B) Ctgf, C) Col1a1, and D) Col6 in mice which consumed indicated HFD for 12 weeks. Expression is normalized to β-actin expression. (n=5; * one-way ANOVA multiple comparisons to SD, # unpaired t-test to SD)
Like VWAT, Tgf-β1 expression was not significantly changed in the SWAT (Figure 17A). However, both Butter and Cocoa diets resulted in increased SWAT expression of Ctgf compared to SD (Figure 17B). Additionally, SWAT expression of Col1a1 was increased compared to SD on the Fish, Palm, Cocoa, and HO Safflower diets (Figure 17C). Finally, Palm, Lard, and HO Sunflower diets resulted in increased expression of SWAT Col6 (Figure 17D). Like the VWAT, this data shows that gene expression is influenced by the dietary fat type and not necessarily by the obesity status of the mouse.

**Discussion**

Dietary fats have been well studied for their impacts on adipose homeostasis and effects on systemic metabolic health\(^\text{151}\). Using a screen of 12 HFDs has invoked many intermediate responses in adiposity and gene expression, allowing for a more nuanced understanding of WAT expansion and function. While our identified correlation between VWAT adiponectin expression and VWAT adipocyte diameter was expected given published findings\(^\text{47,48}\), the depot specificity of this finding to the VWAT was particularly interesting. As female mice lack the depot-specific adipogenic response to HFD observed in male mice\(^\text{131}\), one could study whether adiponectin expression correlates to hypertrophy in both depots in females as well.

The negative correlation between VWAT adiponectin expression and palmitate is consistent with findings that palmitate inhibits adiponectin transcription\(^\text{152}\). However, this publication focused on the study of 3T3-L1 cells *in*
vitro, and therefore the in vivo data presented here represents a valuable contribution to the field. If further work will be needed to determine if these measures are casually linked or are merely correlational. However, a potential hypothesis is that prolonged consumption of palmitic acid is a signal to decrease VWAT adiponectin expression during obesity. Finally, adiponectin is also present in oligomers of varying sizes, and the high molecular weight form of adiponectin is negatively associated to diabetes\textsuperscript{153}. Further work will be needed to determine whether these variations in adiponectin expression also affect dynamics of adiponectin oligomerization.

The identified correlation between VWAT leptin expression and adipocyte formation was unexpected and novel given the expectation that leptin is highly linked to adipocyte size\textsuperscript{138}. While this correlation was surprising, it is consistent with the published observation that plasma leptin levels do not increase until several weeks after HFD consumption\textsuperscript{154}. This time scale would be longer than is needed to alter adipocyte hypertrophy, which can be affected with several hours of fasting and refeeding\textsuperscript{155,156}. In considering how adipose hyperplasia relates to leptin production, two possibilities are that an increase in existing adipocytes results in greater leptin production or that newer adipocytes produce greater quantities of leptin. Another intriguing question would be to interrogate whether adipocyte formation during developmental WAT establishment is a driver of the postnatal surge in plasma leptin\textsuperscript{157}. Interestingly the timing of the postnatal leptin surge (4-12 days of age) roughly corresponds to the initial establishment of the VWAT\textsuperscript{157,158}. 
I identified that VWAT expression of Tnf-α correlated negatively with two measures of hyperplasia—proliferating APs at the onset of diet and BrdU-labeled adipocytes after several weeks of HFD consumption. These data are consistent with the observations that Tnf-α treatment inhibits the differentiation of 3T3-L1 cells into mature adipocytes but provides in vivo findings to support these published in vitro results. As VWAT Tnf-α expression did not correlate to my third measure of hyperplasia, assessed using the AdiCre-ER/mTmG mouse model, this relationship could be driven by AP proliferation and activation. This correlation was only observed in the VWAT, rather than the SWAT, consistent with the male VWAT being a tissue capable of an adipogenic response to HFD. Due to the adipogenic response in female SWAT and VWAT, studying Tnf-α expression in female mice that consumed our HFD screen could distinguish whether this relationship is depot specific or primarily dependent on AP activation.

While these data show that dietary fats impact expression of Tnf-α, IL-6, and IL-1β, no dietary fatty acids correlated with expression of these inflammatory markers. Additionally, although many of these HFDs induced significant fat mass gain in the mice (Figure 1: Dietary fat source determines whether VWAT expands primarily via hypertrophy or hyperplasia. A) VWAT AP proliferation in mice fed SD or pair-fed HFD mice for 7 days. (n=5; unpaired t-test; experiment conducted by Christopher Church) B) AP proliferation in SWAT and VWAT normalized to the SD-fed VWAT proliferation. Mice were fed the indicated HFD for 7 days. (n=5-2; experiment conducted by Jennifer Kaplan) C) VWAT weights of mice which consumed the indicated HFD for 12 weeks (n=4-5; measured by Jennifer Kaplan) D) Quantification of newly
formed adipocytes from AdiCre-ER/mTmG mice. Mice were injected with tamoxifen then
given indicated HFD for 8 weeks. (n=3-18) E) Quantification of VWAT BrdU-labeled
adipocyte nuclei. Mice were given indicated HFD with BrdU water for one week. BrdU
water was then removed, and mice continued to consume indicated diet for a total of 12
weeks. (n=4-5). F) Mean VWAT adipocyte volume from mice which consumed the
indicated HFD for 12 weeks. (n=4-5). Correlations between VWAT mass and G) average
VWAT adipocyte diameter for each diet and H) average rate of adipocyte formation for
each diet. (Significance calculated by one-way ANOVA multiple comparisons to SD or
Spearman correlation) C, Figure 2: SWAT lacks an adipogenic response to HFDs
and expands primarily via hypertrophy. A) SWAT mass after 12 weeks of diet
consumption. (n=5; measured by Jennifer Kaplan) B) Quantification of newly formed
SWAT adipocytes in AdiCre-ER/mTmG mice after 8 weeks of HFD consumption. (n=3-18)
C) Quantification of proportion of BrdU-labeled SWAT adipocyte nuclei in mice which
ate diet for 12 weeks. (n=4-5) D) Mean SWAT adipocyte volume after 12 weeks of
consuming indicated HFD. (n=4-5). Correlations between SWAT mass and E) SWAT
adipocyte diameter and F) SWAT adipocyte formation in AdiCre-ER/mTmG mice.
(Significance calculated by one-way ANOVA multiple comparisons to SD or Spearman
correlation) A), few significantly altered expression of these inflammatory genes.
These results were unexpected, especially as these markers were selected as
likely to be responsive to the obese state. There are varying states of obesity that
are less associated with disease, as observed by the existence of the
“metabolically healthy obese” phenotype. While understanding of this state is
progressing, it importantly indicates that expansion of WAT does not inevitably
lead to tissue dysfunction. Additionally, while the 60% lard HFD (Research Diets,
Inc; D12492) has been extensively studied (as evidenced by thousands of
citations), consumption of animal fats has declined over time\textsuperscript{161}. My data highlights the need to study obesity as it is induced by other fats as these sources may result in differing phenotypes.

Finally, in characterizing expression of fibrotic markers, many of the diets failed to induce a significant change in gene expression, with the exception of the 60\% lard diet when compared directly to the Chow diet. Like the inflammatory gene expression data, the failure of these HFDs to upregulate expression of these fibrotic markers was unexpected given the fat mass gain while consuming these diets, which was expected to drive fibrosis. This observation could be due to the larger quantity of fat in this diet, and perhaps greater changes would be observed if this HFD series contained 60\% fat as well. However, these findings also suggest that further work studying how various dietary fats impact WAT function is needed.

For this study, the use of a series of matched HFDs was instrumental in inducing moderate responses that could be correlated to assess how mechanisms of WAT expansion related to expression of adipokines and markers of inflammation and fibrosis. Future work may focus on the mechanisms of these relationships. Unexpectedly, the expression of several inflammatory and fibrotic genes was not changed during consumption of several of these diets. Further work is needed to understand the connections between dietary fatty acids, obesity, and WAT dysfunction.
Conclusions

The development of the 3T3-L1 cell line enabled the study of signaling and transcriptional pathways involved in adipogenesis\textsuperscript{56,162,163}. Despite this a detailed understanding of the molecular mechanisms driving adipogenesis \textit{in vivo} was lacking. Jeffery et al. provided this necessary \textit{in vivo} study, establishing that diet-induced adipogenesis is specific to the VWAT in male mice and that diet-induced adipogenesis is dependent on Akt2 signaling, in a mechanism specific to obesity\textsuperscript{96}. However, more work was needed to identify the nutritional signal which activated this pathway.

In the work presented here, dietary oleic acid is identified as driver of AP proliferation and differentiation. This role of oleic acid in adipogenesis is noteworthy, given its high prevalence in the Mediterranean diet which is associated with longevity. However, as previously discussed, the Mediterranean diet is associated with many other lifestyle choices which are also associated with improved health, such as high vegetable intake and frequent exercise\textsuperscript{111}. Additionally, expansion of the adipose tissue in the face of increased dietary fat consumption sequesters lipids away from other peripheral tissues where lipid deposition can lead to insulin resistance\textsuperscript{119}. More work is needed to study how dietary oleic acid and its signaling impact metabolic health. This HFD series will continue to be an exciting tool to study these relationships and the connections between other dietary fatty acids and physiology.
Leveraging *in vitro* model with primary APs, we determined that this oleic acid signaling is dependent on Akt2 signaling and activation of lipogenesis. To identify the mechanisms of oleic acid signaling on APs, I initially interrogated the role of the free-fatty acid receptors Gpr120 and Gpr40. Through a variety of experiments, I did not observe any effect of these receptors on AP activation or adipogenesis. These results contrast with published work citing a role for ciliary Gpr120 in the differentiation of 3T3-L1 cells\(^{164}\). Although further work would be needed to characterize the discrepancies between these findings, and while this publication utilizes *in vivo* models to identify the role of cilia in APs for differentiation, the data showing the role for Gpr120 is performed entirely in the 3T3-L1 cell line. While these cells are useful for larger screens and more high-throughput experiments, validation using primary cells or an *in vivo* model would have led to more robust conclusions.

Building on our observation that LXR\(\alpha\) downregulation represents a major change in gene expression during early HFD consumption, I used a combination of *in vitro* and *in vivo* models to demonstrate that oleic acid reduces LXR signaling. In addition, I identified LXR\(\alpha\) phosphorylation as a modulator of *in vivo* AP proliferation during HFD consumption. In using mice with a serine-to-alanine point mutation in LXR\(\alpha\), I observed that VWAT APs demonstrated enhanced diet-induced proliferation, while SWAT APs had increased basal proliferation. Given more time, further work to assess the role of LXR\(\alpha\) phosphorylation on *in vivo* AP differentiation would be valuable. One could determine whether these mice also have enhanced adipocyte formation, either during the SD-fed state or during
HFD consumption. Interestingly, a whole-body knockout of LXRα resulted in increased SWAT leptin expression during SD consumption, consistent with the hypotheses that LXRα plays a role in basal SWAT adipogenesis and that leptin expression is driven by adipocyte formation.

There is also a need to study the role of LXRα in human APs. Activation of LXRα in TH1 cells results in increased expression of aP2, a characteristic protein of adipocytes. The aP2 promoter was also found to contain an LXRα response element\textsuperscript{165}. Little is known about the role of LXRα phosphorylation in human pathology, beyond the observation that phosphorylated LXRα is present in human liver\textsuperscript{166}. Further work is needed to determine the role, if any, of LXRα in human adipogenesis. However, the ability of oleic acid to increase adipogenesis of human APs is promising for further study of this mechanism in human samples.

In addition to mechanisms of adipogenesis, I also used this HFD series to characterize how dietary fats affect expression of a subset of inflammatory and fibrotic markers. While many studies have contrasted a HFD to a low-fat diet, using a series of HFDs provides a more complex context rather than comparing the extremes of lean and obese states. This approach is particularly useful in comparing how mechanisms of WAT expansion are related to gene expression, as I can analyze several intermediate responses, allowing for stronger conclusions. This strategy led to the surprising discovery that VWAT leptin expression is closely correlated to several measures of hyperplasia. Further work will be needed to determine whether this increased leptin expression is due to
more adipocytes present in the tissue or whether the newly formed adipocytes have increased expression of leptin. Additionally, I was surprised by the temporal disparity in the correlation between AP proliferation for a subset of the diets and VWAT leptin expression. Given that AP proliferation is quantified after one week of HFD consumption and leptin expression was quantified after 12 weeks, it suggests that AP proliferation early in HFD may be predictive of eventual leptin expression. However, this relationship is limited to diets that also induced high rates of adipocyte formation, so further studies to understand of WAT response to long-term consumption of the diet will be needed.

Finally, I also used this HFD series to quantify gene expression of a subset of inflammatory and fibrotic markers. This work demonstrates the importance of dietary fats on WAT gene expression and function. Obesity primarily driven by hypertrophy is considered to be linked to WAT inflammation and cell death. However, my data did not find strong correlations between expression of these markers and hypertrophy. While these results could be driven by the lower quantity of fat in my 45% HFD series (the classic HFD contains 60% fat, primarily lard), they also challenge the idea that hypertrophy and inflammation are so consistently linked. While inflammation has been well studied using the 60% lard-based HFD\textsuperscript{167,168}, these HFDs based on alternatives fats provide a different context for the obesity. Given the diverse diet currently consumed by humans and the declining prevalence of lard in the diet \textsuperscript{161}, these findings highlight the need to study obesity as driven by dietary fats other than lard.
The role of fibrosis in obesity is disputed; it is unclear fibrosis provides a check on ballooning adipocyte size or whether it is a marker of a pathological tissue\textsuperscript{45,169}. Like the expression of the inflammatory markers, expression of the fibrotic markers was not significantly increased, even in diets which induced obesity and hypertrophy. However, some of the markers were increased on the lard HFDs, particularly the 60% lard HFD. Again, these findings highlight the need to study obesity in a more physiologically relevant context. While many of the HFDs induced obesity, the mechanisms of WAT expansion (whether primarily through hypertrophy or hyperplasia) differed. Further work would be needed to characterize how WAT gene expression and homeostasis differs on these diets and what effects these differences play in systemic metabolic health.

This work utilized a HFD series to identify a novel mechanism that regulates obesogenic responses and \textit{in vivo} AP proliferation. This study also allowed me to characterize the relationships between obesity and inflammation and fibrosis. These findings further our understanding of the nutritional signals that drive \textit{in vivo} adipogenesis and the relevance of dietary fats to WAT function. Further work is needed to define how these nutritional signals relate to measures of metabolic health and how these concepts can be applied to human health and disease.
Appendix

**Figure 18: Quantification of adipogenesis in female mice.** BrdU pulse-chase performed on female mice. (n=8-10; unpaired t-test) Data published in Jeffery et al. 2016.

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*Percent BrdU positive adipocyte nuclei*
Methods

Animals

All experiments using animals were conducted according to guidelines by Yale University’s Institutional Animal Care and Use Committee (IACUC). Mice used for these studies were on the C57BL/6J background, with wild-type animals purchased from Jackson Laboratories. Adiponectin-CreER/mTmG mice were bred in the Yale Animal Resource Center from Adiponectin-CreER mice gifted by E. Rosen (Beth Israel Deaconess Medical Center, Boston, MA, USA) and mTmG B6.129 (Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J purchased from Jackson Laboratories. Atk2−/− mice were a kind gift from W. Sessa (Yale University, New Haven, CT, USA). PdgfRa/Akt2floxflox mice were bred in the Yale Animal Resource Center from PdgfRa-Cre mice purchased from Jackson Laboratories and Akt2floxflox mice kindly gifted by M. Birnbaum (University of Pennsylvania, Philadelphia, PA, USA) and mediated by K. Martin (Yale University, New Haven, CT, USA). LXRa S196A mice were housed at New York University, and fixed cells were kindly provided by Dr. Michael Garabedian.

Unless otherwise noted, experiments were started using male mice aged 6-8 weeks old. We use the terms VWAT to refer to the perigonadal visceral adipose tissue and SWAT to refer to the inguinal subcutaneous adipose tissue. Standard diet (SD) is purchased from Harlan Laboratories (2018S), and the 60%
lard-based diet was purchased from Research Diets, Inc(D12492). The 45% kcal HFD was purchased from Research Diets with the following catalog numbers:
Table 1: 45% kcal high-fat diets used for screen

<table>
<thead>
<tr>
<th>Fat</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lard</td>
<td>D12451</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>D06022403</td>
</tr>
<tr>
<td>Coconut Oil</td>
<td>D05122301</td>
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<tr>
<td>Fish Oil</td>
<td>D03022403</td>
</tr>
<tr>
<td>Palm Oil</td>
<td>D07081501</td>
</tr>
<tr>
<td>Safflower Oil</td>
<td>D02062102</td>
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<tr>
<td>Butter</td>
<td>D06022405</td>
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<tr>
<td>Cocoa Butter</td>
<td>D11112703</td>
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<tr>
<td>High Oleic Sunflower Oil</td>
<td>D07062503</td>
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<tr>
<td>High Oleic Safflower Oil</td>
<td>D05122103</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>D05042003</td>
</tr>
<tr>
<td>Peanut Oil</td>
<td>D16010705</td>
</tr>
</tbody>
</table>

Fatty acids or tri-oleate (Sigma Aldrich, T7752) were added to the SD to 45% kcal fat. For *in vivo* oleic acid infusion experiments, jugular catheters were implanted into the right vein when mice were 8 weeks old. Oleic acid (20 mM) or vehicle (0.5% fatty acid free BSA in saline) were infused just before the dark cycle for 5 days.

For *in vivo* BrdU experiments lasting one week, mice were given 0.8 mg/mL brdU in drinking water with fresh solution made every other day. For BrdU pulse-chase experiments, BrdU was administered at 0.4 mg/mL in drinking water for one week and then removed and normal water provided.

To treat mice with T0901317, mice were given conditioning injections using vehicle, or medium chain triglycerides (MCT) daily for 4 days. Mice were then given vehicle or 50 mg/kg of T0901317 daily for 3 days, along with HFD before being sacrificed and processed for BrdU analysis.

**AdiponectinCre-ER/mTmG pulse chase experiment**
This experiment was performed as previously described\(^1^9\). In brief, at 8 weeks of age, Adiponectin-CreER/mTmG mice were given daily injections of tamoxifen in vegetable oil at 50 mg/kg for 5 days. The mice were then given a week to recover and then were fed the stated diet for 8 weeks. At the end of 8 weeks, the mice were sacrificed, and their SWAT and VWAT were mounted on slides. The tissues were imaged using confocal microscopy, and the proportion of red to green adipocytes was quantified.

**Immunohistochemistry and adipocyte size quantification**

For the BrdU pulse-chase and adipocyte sizing experiments, adipose tissue was dissected and prepared for paraffin embedding as previously described\(^1^70\). In brief, tissues were dissected and fixed in zinc formalin for 24-48 hours. The tissues were then washed twice in PBS and incubated overnight in 70% ethanol. The tissues were then incubated in 75%, 95%, and 100% ethanol, and Citrisolve (Fisher Scientific, 22143975). The tissues were incubated in melted paraffin before finally being embedded. Paraffin blocks were sectioned by Yale Pathology Tissue Services.

For adipocyte sizing, tissue sections were stained with Masson’s Trichrome stain and imaged using by Yale Pathology Tissue Services. To quantify adipocytes sizes, sections were systemically imaged and processed using a Cell Profiler pipeline adapted from a previous publication \(^1^70\). At least 300 adipocytes were quantified from each mouse. To correct for the fact that imaging random cross sections of the roughly spherical adipocytes was not likely to
capture the adipocyte at its greatest diameter, a correcting program was kindly written by Dr. Taylor Malone at Yale University. This program was adapted from the program StripStar, which calculates a corrected distribution of spheres from a measured distribution of sectional areas. The original StripStar program developed for rock grain-size. The histogram of observed cell radii is compared to a theoretical distribution where cells sizes are uniformly distributed. The corrected histogram is calculated starting from the largest observed cell size, iteratively "stripping off" observed cells until all cells are accounted for. A complete description of the calculation can be found in Heilbronner et. al\textsuperscript{171}.

For the BrdU pulse-chase experiment, sections were deparaffinized as previously published \textsuperscript{170}. In brief, the slides were incubated in Citrisolve, 100%, 95%, 70% ethanol and PBS. Sections were surrounded with a PAP pen and blocked with a 2% BSA in PBS solution. Sections were stained using rat anti-BrdU (Abcam, 6326; 1:300) and rabbit anti-caveolin-1 (Cell Signaling 3238 1:400) and washed three times in PBS. Sections were then stained with anti-rabbit Rhodamine-X-Red (Jackson Immunoresearch, 111-295-144; 1:250) and anti-rat Alexa Fluor 488 (Jackson Immunoresearch, 112-545-167; 1:250) secondary antibodies and washed three times in PBS. DAPI Fluoromount-G mounting media (Southern Biotech) was then used, along with a coverslip which was sealed with nail polish. Sections were then imaged with confocal microscopy.

To quantify phospho-LXRα in APs, sections were deparaffinized as described. After blocking with 2% BSA in PBS, cells were incubated overnight
with rat anti-Sca1 (BioLegend, 122501; 1:50) and rabbit anti-phospho- LXRα (Garabedian lab, NYC; 1:50) at 4°C. Sections were washed and incubated with donkey anti-rat Alexa Fluor 647 (Jackson Immunoresearch, 1:800) for 2 hours at room temperature. Seconds were then washed three times in PBS. Tyramide SuperBoost Kit Alex Fluor 594 was used to augment the phosphor- LXRα signal (ThermoFisher Scientific, B40944). Sections were incubated with poly-HRP-conjugated secondary antibody at room temperature for 1 hour. They were then washed three times with PBS and incubated with tyramide working solution for 5 minutes at room temperature. Sections were washed three times with PBS when incubated at room temperature with DAPI (1:1000) for 15 minutes. They were then washed three times with PBS and mounted for imaging.

**Flow cytometry**

Flow cytometry for analysis, isolation of APs, and BrdU quantification were performed as previously described. In brief, adipose tissue was dissected, minced, and digested using a 0.8 mg/mL collagenase II (Worthington Biochemical Corporation LS004176) dissolved in a solution of 3% BSA in Hank’s Balanced Salt Solution (HBSS; Sigma Aldrich H8264). Cells were placed in a shaking water bath at 37°C for one hour, shaken by hand, then continued to shake for 15 minutes. If cells were being sorted, they were first filtered through a 100 μm filter. Cells for all experiments were filtered through a 40 μm filter. If cells were being analyzed or sorted, they were stained using antibodies for CD45 APC-eFluor780 (eBioscience, 47-0451-80; 1:1000 for sorting, 1:500 for BrdU
analysis), CD31 PE-Cy7 (eBioscience, 25-0311-82; 1:500), CD29 Alexa Fluor 700 (BioLegend, 102218; 1:400), CD34 Alexa Fluor 647 (Biolegend, 119314; 1:400), Sca1 Pacific Blue (BD Biosciences, 560653; 1:400), and CD24 PerCP-Cyanine 5.5 (eBioscience, 45-0242-80; 1:250) for 20-30 minutes on ice. Cells were sorted with a BD FACSAriaII and analyzed using a BD LSRII analyser and BD FACS Diva software.

If the cells were being analyzed for BrdU quantification, after digestion and filtration, they were prestained with antibodies for CD45 APC-eFluor780, CD31 PE-Cy7, CD29 Alexa Fluor 700 (as listed above), and Sca1 V500 (BD Horizon, 561228; 1:500) for 20-30 minutes on ice. Cells were then washed, fixed, and permeabilized according to the instructions for Phosflow Lyse/Fix and Perm Buffer III (BD Biosciences). Cells were then treated with Dnase (Worthington Biochemical Corporations, LS002007) in PBS and washed with 3% BSA in HBSS. The cells were incubated with anti-BrdU Alexa Fluor 488 or 647 (Phoenix Flow Systems, AX647 or AX488; 1:30) overnight, washed, then stained a mixture of antibodies for CD45, CD31, CD29 (as listed above), CD34 Alexa Fluor 647 (listed above) or Brilliant Violet 421 (BioLegend, 119321; 1:400), Sca1 Pacific Blue or V500, and CD24 (1:200) for 30 minute at room temperature. Cells were then washed and analyzed.

To quantify proliferation in LXRα S196A mice, Ki67 was used as a marker of proliferation due to BrdU being a restricted substance in the New York University mouse facility. To quantify Ki67 in APs, after antibody staining, WAT was digested and incubated in Phosflow Lyse/Fix as discussed for the BrdU
quantification. Cells were then washed twice in Intracellular Staining Permeabilization Wash Buffer (BioLegend, 421002). After washing, cells were incubated with Anti-Ki67 FITC (eBioscience, 11-5698-80; 1:50). Cells were washed twice again in Permeabilization Wash buffer and resuspended in 3% BSA in HBSS for analysis.

**Isolation of human APs**

Consent and experimental protocols to isolate human APs were reviewed and approved by the Yale Internal Review board (HIC protocol number 1109009063). Samples were subcutaneous and abdominal adipose tissue regarded as waste materials from bariatric surgeries or elective abdominoplasties. Samples were stored in sterile saline solution until processing.

Tissues were washed with Krebs Ringer Phospoate (KRP) solution containing 0.127 M NaCl, 5 mM KCl, 2 mM sodium phosphate dibasic, and 8 mM sodium phosphate monobasic. Tissues were minced, washed in KRP solution, and centrifuged to separate red blood cells. Samples were then digested in KRP solution with 3% FBS and collagenase type II for 75 minutes in a shaking water bath at 37ºC. Samples were then filtered and washed with KRP and 3% chelexed fetal calf serum (FCS). The floating layer of adipocytes was removed. Remaining cells were then stained using antibodies for CD235 PE-Cy5 (BioLegend, 306605), CD45 Pacific Blue (Biolegend, 368539), GP38 PE (BioLegend, 337003), CD13 APC-Cy7 (BioLegend, 301709), CD34 APC (eBioscience), CD90 (BD Biosciences). Cells were then washed with KRP with 3% chelexed FCS.
Cells were then selected for GP38 using EasySep Human PE positive selection kit (Stem Cell Technologies, 17664). Cells were incubated with the PE selection cocktail for 15 minutes, then incubated with the magnetic beads and placed in the magnet. Cells were eluted up to 2 times using KRP with 3% chelexed FCS. Cells were then sorted using the following marker scheme: CD45-; CD235-, CD90+, GP38+, and CD34+.

Lipidomic analysis of diet, plasma and WAT

Plasma and WAT for fatty acid analysis was harvested at 6am after ad libitum feeding. Samples were analyzed at the Mouse Metabolic Phenotyping Center at the University of Cincinnati. Samples were saponified and methylated for analysis of GC mass spectrometry. Retention times were compared to known standards. Fatty acid methyl esters were separated and quantified by gas-liquid chromatography with a Hewlett-Packard 68900 series II gas chromatograph, using an Omegawax column (Supelco) and flame ionization detector.

Cell transplant assay

Cell transplants were performed as previously described\textsuperscript{172}. In brief, SWAT and VWAT APs were harvested and pooled from several mice as detailed above. APs were rinsed in PBS and resuspended in PBS. Recipient mice were anesthetized with Isothesia (Covetrus, 029405) when aged 4 weeks old. At least 0.5 million APs were injected into the tip of one recipient VWAT depot. Recipient
mice were allowed to recover for 2 weeks and then placed on HFD for 1 week. They were then sacrificed and analyzed for BrdU incorporation into APs.

In vitro differentiation of primary APs and 3T3-L1 cells and Oil Red O assay

Primary APs were isolated as described above. They were cultured in growth media consisting of DMEM (ATCC, 30-2002) supplemented with 1% penicillin/streptomycin (Sigma Aldrich, G1146) and 10% fetal bovine serum (Life Technologies). Upon reaching confluence, media was not changed for 48 hours. To differentiate the APs, the media was supplemented with 0.1 μg/mL insulin (Sigma Aldrich I-5500) and maintained in this solution for 7 days with media changings every other day. If cells were being differentiated in the presence of a fatty acid, the fatty acid was conjugated to fatty acid free BSA (Sigma Aldrich 7030) and added to the differentiation media at 100 μM.

3T3-L1 cells were maintained in the same growth media as primary APs. They were passaged when nearly confluent by rinsing with PBS and detaching with 0.05% Trypsin/EDTA (Life Technologies, 25300054). When nearly confluent, experimental cells were differentiated with an adipogenic cocktail (MDI) consisting of 0.1 μg/mL insulin, 30 μg/mL 3-isobutyl-1methylxantine (Sigma Aldrich, 15879), and 0.1 μg/mL dexamethasone (Sigma Aldrich, D-4902). After 48 hours, media was maintained in 0.1 μg/mL insulin and changed every other day for 7 days.

To quantify accumulated lipid, cells were washed twice with PBS and fixed in a solution containing 0.2% glutaraldehyde and 2% formaldehyde in PBS. The
cells were then washed twice with PBS, twice with water, and briefly with 60% isopropanol. After the washes, the cells were stained with a mixture of 60% Oil Red O (Electron Microscopy Sciences 26503-02) and 40% water and then washed briefly with 60% isopropanol and twice with water. The cells were imaged and left in water overnight. The next day, the cells were dried for several hours and then the dye was extracted using a solution of 4% NP40 in isopropanol. The absorbance of the extraction solution was then analyzed on a spectrophotometer at 500 nm.

Primary APs treated with inhibitors were treated 2 hours before differentiation. Cells were treated with Glucose Transporter Inhibitor II (Calbiochem, 10 µM) or C75 fatty acid synthase inhibitor (Sigma Aldrich; 100 µM). Cells were treated with 5 µm T0901317 in DMSO.

**Transfection and luciferase reporter assay**

3T3-L1 cells were cultured in a 48 well plate to nearly confluent before transfection. Cells were transfected using Lipofectamine 2000 Transfection Reagent (LifeTechnologies, 11668019) in OptiMEM (LifeTechnologies, 31985070). Cells were transfected with Cignal LXR Reporter Kit (Qiagen, 336841; 500 ng) for 24 hours. Media was then changed to treatment media.

LXR activity was quantified using the Luciferase Assay System (Promega, E1500) according to the protocol. In brief, cells were lysed and spun at 12,000 g for 2 minutes at 4°C. Sample was then mixed with luciferase assay substrate, and luminescence was quantified over 10 seconds.
**Isolation of RNA and real time qPCR**

If analyzing gene expression in tissue, tissues were dissected, frozen in liquid nitrogen and stored at -80°C. The tissues were then homogenized in Trizol LS (Life Technologies 10296010). If analyzing gene expression in cells, cells were washed in PBS and then suspended in Trizol LS. If not being processed immediately, the cells were frozen in Trizol LS at -80°C.

RNA from both tissues and cells was harvested using a Direct-zol RNA Miniprep kits (Zymo Research, R2051) and quantified using a nanodrop spectrophotometer. RNA was converted to cDNA using a cDNA Reverse Transcription kit (Applied Biosystems, 4368814). qPCR was performed with KAPA SYBR FAST for LighCycler480 (Kapa Biosystems KK4611) on a LightCycler 480 Real Time PCR system (Roche).

**Immunoblots**

For Akt2 immunoblots, primary APs were lysed using 1% IGEPAL with protease and PhosStop phosphatase inhibitors (Roche). For LXRα immunoblots, protein was lysed using a mixture of SDS (0.645%), sodium deoxycholate (6.45%), Triton X100 (6.45%) supplemented with 11.5 mM sodium pyrophosphate, 11.5 mM β-glycerophosphate disodium, and protease and PhosStop phosphatase inhibitors. Protein was quantified using Pierce BCA protein assay kit (Life Technologies, 23225). Protein was incubated with NuPage 2X Sample Buffer (ThermoFisher Scientific, NP0007) and Sample Reducing
Agent (ThermoFisher Scientific, NP0009). Western blots for Akt2 were run on 10% polyacrylamide gels, while Western blots for LXRα were run on 10% Bis-Tris gels. Protein was then transferred to PVDF membranes using the Invitrogen NuPage system.

For Akt2 immunoblots, membranes were stained with rabbit anti-Akt2 (Cell Signaling, 3063; 1:1000) or rabbit anti-phospho-Akt2 (Cell Signaling, 8599; 1:500) in tris-buffered saline with 0.1% Tween-20 (TBST). Membranes were then incubated with goat anti-rabbit-HRP secondary antibody (Jackson Immunoresearch, 211-032-171; 1:10,000) and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

For LXRα immunoblots, membranes were blocked with 5% BSA TBST. They were incubated with rabbit anti-phospho- LXRα kindly provided by Dr. Michael Garabedian’s lab at New York University (1:2000) or rabbit-anti LXRα. They were then incubated with goat anti-rabbit-HRP (ThermoFisher Scientific, MA5-32691; 1:500) and developed.
References:


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